ENZYMATIC PREPARATION OF MICROBIAL POLYESTER-BASED NOVEL BLOCK COPOLYMERS AS THERMOPLASTIC BIOMATERIALS

DAI SHIYAO

NATIONAL UNIVERSITY OF SINGAPORE

2009

ENZYMATIC PREPARATION OF MICROBIAL POLYESTER-BASED NOVEL BLOCK COPOLYMERS AS THERMOPLASTIC BIOMATERIALS

DAI SHIYAO

(*M. Eng., Zhejiang University, China*)

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY

DEPARTMENT OF CHEMICAL & BIOMOLECULAR ENGINEERING

NATIONAL UNIVERSITY OF SINGAPORE

2009

To My Parents

Acknowledgements

First of all, I would like to express my sincere gratitude to my supervisor, Professor Li Zhi, for his patience and continuous support to me. Whenever I encountered a problem, he has always been there for me. Besides helping me to solve the problems and sharing his inspiring ideas, the most important thing he makes me understand is how to do research. He also seizes every tiny problem and tries to solve it promptly. Such kind of critical attitude and rigorous schclorship in research will acoompany me in the rest of my life.

This thesis has benefited by many other people's efforts. I would like to acknowledge Professor Hong Liang and Dr. Liu Bin for their helpful advices and discussion. The kind helps from Mdm. Li Fengmei, Mdm. Li Xiang, Ms. Chew Su Mei, Mdm. Han Yanhui, Mr. Wong Chee Ping, Mr. Cheung Augustine, Dr. Dharmarajan Rajarathnam, Mr. Ng Kim Poi, Mr. Boey Kok Hong, Ms. Tay Kai Si, and Ms. Xu Yanfang, are really appreciated. Without their help, this thesis would never have been so sucesessful. The financial support provided by National Univeristy of Singapore was also grarefully acknowledged.

Additional thanks go to my colleagues, Ms. Zhang Wei, Ms. Xue Liang, Dr. Christine Schutz, Ms. Tang Weng Lin, Dr. Wang Zunsheng, Dr. Xu Yi, Mr. Jia Xin, Ms. Wang Wen, Mr. Mojtaba Binazadeh, Mr. Pham Quang Son, Ms. Ngo Nguyen Phuong Thao, and Dr. Mou Jie, for their friendship and valuable discussion during the study. Special thanks go to three of my best friends, Dr. Liu Dongming, Dr. Liu Changkun and

Dr. Chen Yongzheng, for bringing me a radiant life in the past four years. I also would like to thank my other friends, Ms. Bi Xinyan, Ms. Tian Xiaoning, Ms. Liu Jiajia, Ms. Peng Na, Ms. Xue Changying, Mr. Pan Jie, Mr. Hu Zhongqiao, Mr. Yang Jinhua, Mr. Deng Da, Mr. Wang Yusong, Mr. Wu Xuesheng, Mr. Li Ya, and Dr. Li Shi. I really spent a great time with all of you.

Last but not least, I would like to express my gratitude from the bottom of my heart to my parents and brother. Thank you very much for your continuous and invaluable support in my life. I could not finish the whole study without the great love and care from you.

TABLE OF CONTENT

Summary

Microbial poly[(*R*)-3-hydroxyalkanoate]s (PHAs) are biodegradable and biocompatible materials with applications in several biomedical fields. However, their application as thermoplastic biomaterials is limited due to the poor thermoplastic properties. The main purpose of this PhD project is to develop novel polymeric materials for biomedical applications by enzymatic modification of microbial poly[(*R*)-3-hydroxybutyrate] (PHB). More specifically, this project aims to prepare thermoplastic block-copolymer containing PHB as hard segment and enzymatically synthesized polymer (polyesters or polycarbonate) as soft segment, to achieve desired properties and develop novel and general enzymatic method for the synthesis of *di*-, *tri*-, and multiple block copolymers.

Firstly, enzymatic modification of PHB was achieved by the ring-opening polymerization (ROP) of ε-caprolactone (CL) using low-molecular weight telechelic hydroxylated poly[(*R*)-3-hydroxybutyrate] (PHB-diol) as initiator and Novozym 435 (immobilized *Candida antarctica* Lipase B) as catalyst in anhydrous 1,4-dioxane or toluene. The reaction was investigated at different conditions with two different types of PHB-diols: PHB-diol(P) containing a primary OH and a secondary OH end groups; and PHB-diol(M) consisting of 91% PHB-diol(P) and 9% PHB-diol containing two secondary OH end groups. The ROP of CL by using PHB-diol(M) (M_n of 2380, NMR) as initiator at the molar ratio of 50:1 under the optimal conditions in 1,4-dioxane gave the

corresponding poly[HB(56wt%)-*co*-CL(44wt%)] with M_n (NMR) of 3900 in 66% yield. Polymerization of CL and PHB-diol(P) (M_n of 2010, NMR) at the same conditions in toluene gave the corresponding poly[HB(28wt%)-*co*-CL(72wt%)] with M_n (NMR) of 7100 in 86% yield. Both polymers were characterized by ^{1}H- and ^{13}C-NMR and IR analyses as *di*-block co-polyesters containing a PHB block with a secondary OH end group and a poly(ε-caprolactone) (PCL) block with a primary OH end group. NMR analyses and control experiments suggested no formation of random copolymers and no change of the PHB block during the reaction. The enzymatic ring-opening polymerization was selectively initiated by the primary OH group of PHB-diol, whereas the secondary OH group remained as an end group in the final polymers. The thermal properties of the *di*-block poly(HB-*co*-CL)s were analyzed by DSC, with improved T_g values for the elastomer segment: poly[HB(56wt%)-*co*-CL(44wt%)] with M_n (NMR) of 3900 demonstrated a T_g of -57°C, T_m of 145, 123, and 53 °C; and poly[HB(28wt%)-*co*-CL(72wt%)] with M_n (NMR) of 7100 gave a T_g of -60°C, T_m of 147 and 50 °C. Thus, the selective enzymatic ring-opening polymerization with PHB-diol as macro-initiator provides a new method for the preparation of PHB-based block copolymers as biomaterials with good thermoplastic properties and novel structures containing functional end groups.

To achieve better plastic and elastic properties of PHB-based copolymers, poly(ester-carbonate)s were synthesized by the ROP of trimethylene carbonate (TMC) with PHB-diol, and poly(HB-*co*-CL), respectively, as initiator and Novozym 435 as catalyst in anhydrous 1,4-dioxane or toluene. PHB-diol initiated ROP of TMC gave the

corresponding *di*-block poly(ester-carbonate)s with M_n (GPC) of 4400-8700 in 57-89 % yield, while the *tri*-block poly(ester-carbonate)s prepared from poly(HB-*co*-CL) intitaed ROP of TMC showed M_n (GPC) of 7900-9600, and yield of 60-66 %. The polymer structures were characterized by ^{1}H-NMR as block co-poly(ester-carbonate)s containing a PTMC block as soft domain and PHB or PCL block as hard domains. Different initiators with different type of OH end groups resulted in *di*- or *tri*- block copolymers, respectively. The thermal properties of the block poly(ester-carbonate)s were analyzed by DSC, with excellent T_g values for the soft domain ranged from -20 to -48 ^oC. The poly(ester-carbonate)s were further polymerized with MDI to prepare the corresponding poly(ester-urethane)s (PUs). Mechanical test of the PU prepared from poly(HB-*co*-TMC) with HB:TMC weight ratio of 26:74 showed a Young's modulus of 23 MPa, the maximum stress of 6.37 MPa and elongation at break of 252%. The results of PU prepared from poly(HB-*co*-CL-*co*-TMC) with HB:CL:TMC weight ratio of 14:25:51 had a Young's modulus of 18 MPa, the maximum stress of 8.30 MPa and elongation at break of 304%, thus being a plastic-elastmer. The selective enzymatic ring-opening polymerization of TMC with PHB-diol as initiator provides a new method for the preparation of block copoly(ester-carbonate)s with novel structures containing functional end groups as biomaterials. Moreover, the thermoplastic properties and mechanical properties can be easily controlled by changing the feed ratio of macro-diols to achieve the different ratio of HB / CL / TMC blocks.

A novel and general method for block copolymer synthesis based on polycondensation was also developed. For the first time, thermoplastic copolyesters containing PHB and poly[(*R*)-3-hydroxyoctnoate] (PHO) blocks were enzymatically

prepared by one- or two-step lipase-catalyzed polycondensation using telechelic macro-diols as starting materials. Reaction of PHB-diol (M_n of 3100, GPC), PHO-diol (M_n of 3200, GPC), and divinyl adipate at 1:1:2 ratio in the presence of Novezym 435 gave the corresponding block copolyesters poly(HB-*co*-HO) (M_n of 9800, GPC) in 55% yield. In the two-step polycondensations, PHB-diol was first reacted with 10 fold of divinyl adipate with Novezyme 435 as catalyst to give 73% of PHB containing two vinyl esters end groups (M_n of 2700, GPC); the PHB-vinyl ester was further reacted with PHO-diol at a ratio of 1:1-2 in the presence of Novezyme 435 to give block poly(HB-*co*-HO)s (M_n of 8800-14200, GPC) in 55-62% yield. The polymer structures were analyzed by ^{1}H NMR, and the thermal properties were measured by DSC. The enzymatically prepared block copolymer poly(HB-*co*-HO)s demonstrated T_m of 136 to 153 ^oC and T_g of -37 to -39 ^oC, being potentially useful thermoplastic biodegradable and biocompatible materials.

List of Tables

List of Figures

Nomenclature

CALB	*Candida antarctica* Lipase B
-*co*-	- copolymer of -
DMF	Dimethylformamide
DMSO	Dimethylsulfate
DSC	Differential Scanning Calorimeter
GPC	Gel Permeation Chromatography
Lipase CA	*Candida antarctica* lipase
Lipase CR	*Candida rugosa* lipase
Lipase CC	*Candida cylindracea* lipase
Lipase PC	*Pseudomonas cepacia* Lipase
M_n	Number Average Molecular Weight
M_w	Weight Average Molecular Weight
NMR	Nuclear Magnetic Resonance
PCL	Poly(ε-caprolactone)
PDI	Polydispersity Index
PFL	*Pseudomonas fluorescens* Lipase
PHA	Polyhydroxyalkonoate
PHB	Polyhydroxybutyrate
PHO	Polyhydroxyoctanoate
PPL	*Porcine pancreatic lipase*
PTMC	Poly(trimethylene carbonate)

ROP	Ring-Opening Polymerization
T_g	Glass Transition Temperature
T_c	Crystallinity Temperature
T_m	Melting Temperature
WAXD	Wide Angle X-ray Diffraction

CHAPTER 1

INTRODUCTION

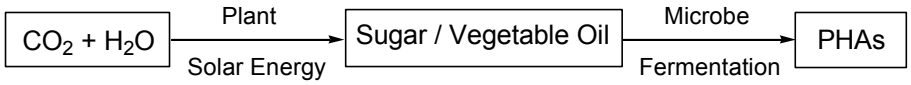
A biomaterial is a synthetic or natural material used to prepare part of a living system or to function in intimate contact with living tissue. Biomaterials play important roles in the treatment of diseases and improvement of health during history. Early biomaterials including metals, woods and nacres were used in dentistry over 2000 years ago, the linen sutures and metallic sutures were also recorded by early Egyptians and Europeans. Generally, the current available biomaterials can be classified into four types: metallic biomaterials, ceramic biomaterials, composite biomaterials, and polymeric biomaterials. Metallic biomaterials such as Ti, Ti alloys, Mg, Mg alloys and Co-Cr-Mo alloys, are suitable for load-bearing applications due to their combination of high mechanical strength and fracture toughness. For example, they play essential roles in the repair, replacement or fixation of diseased or damaged bone tissue. Ceramic biomaterials are usually *non*-toxic, inert, and chemically or thermally stable, they are mainly used in the applications where biocompatibility and thermal stability requirements are essential. Alumina (Al_2O_3), yttria stabilized zirconia (ZrO_2) and Bioglass® are commonly applied ceramic biomaterials and demonstrate excellent biocompatibility. Although metallic and ceramic biomaterials have wide application in biomedical fields, the mechanical and physical properties of these materials are still limited due to their specific structures, and can only be modified in a very small range. This limitation prevents them from fitting the variable requirements of biomedical applications. Alternatively, composite biomaterials were studied to provide with improved physical or mechanical properties for specific applications. Composite biomaterial usually refers to a biomaterial consisting of two or more chemically distinct constituents that are able to act synergistically to give properties superior to those provided by either component alone. A few composites of metallic and

ceramic biomaterials provide significantly improved mechanical and physical properties and are applied as new biomaterials.

Besides metallic, ceramic, and composites biomaterials, polymeric biomaterials, or biopolymers, represent the largest class of biomaterials. With different repeat units in backbone and different chain lengths, the physical properties and mechanical properties of polymers can be adjusted in a wide range. Furthermore, based on the development of synthetic technology, new polymers can be designed and prepared for specific biomedical applications nowadays. Biopolymers are widely involved in medical devices, such as orthopedic, dental, soft tissue, and cardiovascular implants. From the origin of biopolymers, they can be classified into petroleum-based synthetic biopolymers and microbial-based natural polymers. Synthetic polymers provide with quite different structures and properties, but only limited polymer candidates can be selected as biopolymers with desired biocompatibility, which was defined as the ability of a material to perform with an appropriate host response in a specific application. Natural polymers are produced in *vivo* by a large amount of microorganisms. The relatively limited amount makes them much more expensive and scarce than synthetic biopolymers. However, natural polymers have great advantages in biocompatibility compared with synthetic biopolymers. Among the class of natural polymers, poly[(*R*)-3-hydroxyalkanoates] (PHAs) are important members, which attracts great research interests for a few decades due to their significant biocompatibility and thermoplastic properties.

1.1 Poly[(*R*)-3-hydroxyalkanoate]s (PHAs) as Biomaterials

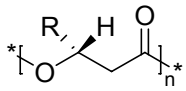
PHAs are produced by a wide variety of microorganisms as carbon storage materials.[1, 2] Being an important family of natural products, PHAs are biorenewable and do not depend on the supply of petroleum, thus have great advantages over synthetic polymers. Microbial PHAs have molecular weights of up to 3,000,000 Daltons and are generally thermo-processable, which make them be competitive as biomaterials for applications in both conventional medical devices and contemporary tissue engineering. The monomer compositions of PHAs are variable and can be manipulated by changing the carbon sources and the growth conditions of microorganisms. More than 100 structures of PHAs have been reported.



Scheme 1.1 Production of PHAs

So far, several PHA-members including poly [(*R*)-3-hydroxybutyrate] (P3HB), poly [(*R*)-4-hydroxybutyrate] (P4HB), poly [(*R*)-3-hydroxybutyrate-*co*-hydroxyvalerate] (PHBV), poly [(*R*)-3-hydroxyhexanoate] (PHHx), poly [(*R*)-3-hydroxyoctanoate] (PHO) is produced in sufficient quantity for research applications, and PHB and PHBV are commercially available in large scale. Over the past few decades, PHAs and its composites have been widely studied and applied to produce biomedical devices

including sutures, suture fasteners, meniscus repair devices, rivets, tacks, staples, screws, bone plates and bone plating systems, surgical mesh, repair patches and so on. [3, 4]



P(3HA)

R=hydrogen	3-hydroxypropionate (3HP)
R=methyl	3-hydroxybutyrate (3HB)
R=ethyl	3-hydroxyvalerate (3HV)
R=propyl	3-hydroxycaproate (3HC)
R=butyl	3-hydroxyheptanoate (3HH)
R=pentyl	3-hydroxyoctanoate (3HO)
R=hexyl	3-hydroxynonanoate (3HN)
R=heptyl	3-hydroxydecanoate (3HD)
R=octyl	3-hydroxyundecanoate (3HUD)
R=nonly	3-hydroxydodecanoate (3HDD)

Scheme 1.2 Representitive Members of PHAs

1.2 PHB as biomaterials

Poly[(*R*)-3-hydroxybutyrate] (PHB) is the most prominent member of the PHA family, and it was first mentioned in literature as early as 1901. The detailed studies were firstly reported by Maurice Lemoigne since 1925.[5-8] He showed that PHB is a polyester with an empirical formula $(C_4H_6O_2)_n$ and he also reported the differences in melting points with different degree of polymerization (DP) of PHB. Over the next 30 years, PHB was mainly studied as an academic curiosity and the related research work was reviewed by Williamson and Wilkinson.[9] With the development in production technology of PHB and PHBV, a variety of applications of PHB and PHBV were explored, especially in biomedical applications due to its good biocompatibility and biodegradability.

1.2.1 Production of PHB

Many aerobic and anaerobic bacterial species, under nutrient limiting conditions with a sufficient supply of carbon, accumulate submicron inclusion bodies composed of PHAs, and the most prevalent of these products is PHB. PHB inclusion bodies are normally spherical, about 0.5 μm in diameter.[10] PHB plays the role of an electron sink for the excess reducing power developed in an aerobic bacterium under conditions of oxygen limitation. Figure 1.1 shows the bacterial cells packed with granules of PHB. The amount of PHB in bacterial cells is normally 1-30% of their dry weight; however, some *Azotobacters* and *Alcaligenes* species can accumulate up to 90% of their dry biomass.[10-13]

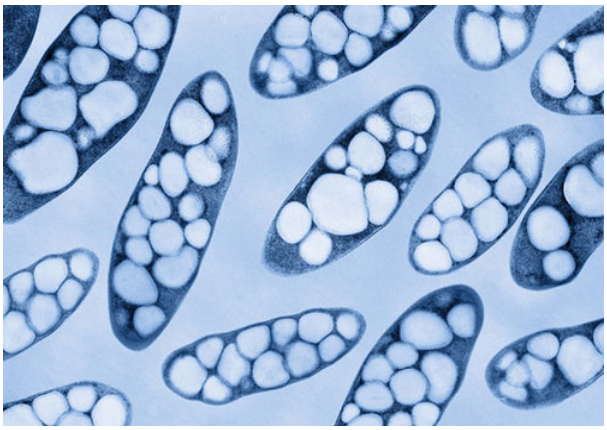


Figure 1.1 Transimission electron micrograph of bacterial produced poly(3-hydroxybutyrate) inclusion bodies. -photo *via* inhabitat.com

With the rapid development of fermentation technologies in 1970s, Imperial Chemical Industries (ICI) in U.K. began the commercialization of PHB. In 1982, they developed a process for industrial production of PHB with the bacterial *Alcaligenes*

eutrophus grown on glucose, which may produce up to 70% of its dry biomass as PHB. They also applied a patent of a procedure to produce copolymer from β-hydroxybutyrate and β-hydroxyvalerate, which is known as PHBV or Biopol® and the first commercial product made of Biopol® was launched in Germany in 1990.[14]

Although PHB was discovered as a natural polymer, it can also be synthesized by chemical route via the ring-opening polymerization of β-butyrolactone. Chemical synthesis of PHB is a competitive route to the bacterial fermentation. It can produce PHB in low-cost and large-scale, but there is a chiral center in the ring structure of β-butyrolactone, and the ability to control the stereochemistry of polymerization is essential if the analogue of the natural polymer is to be made. Production of isotactic PHB is chemically possible if a single isomer of the monomer is used in the reaction, but the single-isomer raw material for synthesis of PHB is relatively expensive and can not be widely applied in industry-scale production.[1, 2]

1.2.2 Physical properties of PHB

PHB is known as the most prominent polyester in PHAs family. It is produced in large scale by a wide variety of microorganisms, and exists in the cytoplasmic fluid in the form of granules with diameters in 0.3-1.0 μm.[1] PHB is a linear saturated polyester with high molecular weigh (approximately 10^5-10^6) behaving as a conventional thermoplastic material with a melting temperature (T_m) of 175-180°C and glass transition temperature (T_g) of 4°C.[1, 15-17] Owing to the enzymatic synthesis, PHB has an exceptional stereochemical purity, and the chiral centers all possess the *R* stereochemical

configuration,[18] which implies that this polymer is completely isotactic and its crystallinity is more than 50%. The suitable crystallinity usually leads to enhanced mechanical properties, unique thermal behavior, and increased fatigue strength. These properties make semi-crystalline polymer desirable materials for biomedical applications.

The physical and mechanical properties of PHB and some common polymers are compared and summarized in Table 1.1.[1] The Young's modulus (3.5 GPa) and the tensile strength (40 MPa) of PHB film are similar to those of isotactic polypropylene, while the elongation at break (6%) is much lower than that of polypropylene (400%). Moreover, the glass transition temperature (T_g) of polypropylene (-10 ^oC) is also lower than that of PHB (4 ^oC).

Table 1.1 Physical and Mechanical Properties of PHB and some common polymers.[1]

Properties	P(3HB)	Polypropylene	Poly(ethylene terephthalate)	Nylon-6,6
T_m (^oC)	180	176	267	265
T_g (^oC)	4	-10	69	50
Crystallinity (%)	60-80	50-70	30-50	40-60
Density (g/cm^3)	1.250	0.905	1.385	1.14
Water Uptake (wt%)	0.2	1.7	2.9	2.8
Young's Modulus (GPa)	3.5	0.0	0.4	4.5
Tensile Strength (MPa)	40	38	70	83
Extension at Break (%)	6	400	100	60

ICI developed a procedure to produce copolymer of PHB and PHV, known as PHBV or Biopol®. It has much improved physical and mechanical properties than those of PHB. Compared with the original PHB, the flexibility and toughness of PHBV are greatly improved by incorporating PHV units. The change of PHA compositions also

allow favorable mechanical properties, biocompatibility, and degradation times within desirable time frames under specific physiological condition.[19, 20] With different component contents of HV units, the melting temperature can be modified from 175 to 97°C, but the degradation temperature is less affected.

Table 1.2: Polymer Properties Comparison: PHB and PHB/HV Compared with Conventional Plastics.[21]

	T_m °C	T_g °C	Young's Modulus GPa	Tensile Strength MPa	Notched Izod J/m
PHB	179	10	3.5	40	50
PHBV(3% HV)	170	8	2.9	38	60
PHBV(9% HV)	162	6	1.9	37	95
PHBV(14% HV)	150	4	1.5	35	120
PHBV(20% HV)	145	-1	1.2	32	200
PHBV(25% HV)	137	-6	0.7	30	400

1.3 Objectives

As mentioned in previous sections, the main problems existed in current biomaterials research are the following:

1. There are only limited synthetic biopolymers with acceptable biocompatibility or biodegradability to favor the various requirements for biomedical applications.

2. Microbial polyester PHAs are potentially useful biomaterials, but the physical and mechanical properties are not so desirable.

3. Preparation of block copolymers is an effective way for the improvement of polymer properties. However, the synthesis of biomaterials often involves the use of toxic chemical catalysts, which may cause conflict with the desired biocompatibility.

4. Enzymatic polymerization is *non*-toxic and can provide good biocompatibility of the prepared polymers, but the methods for the preparation of block copolymers are rather limited.

Thus, the general objective of this thesis is to develop novel polymeric biomaterials for biomedical applications by enzymatic modification of microbial polyester to achieve controllable physical and mechanical properties. More specifically:

1. Novel block copolymers will be designed and prepared as biomaterials, with PHB as hard domain and other polyesters or polycarbonates materials as soft domains, to provide controllable and desired physical, mechanical and plastic or elastic properties.

2. Novel enzymatic synthetic methods will be established to prepare block co-polyesters or co-poly(ester-carbonate) *via* ring-opening polymerization. Oligomer PHB-diol will be used as macro-initiator for the enzymatic ring-opening polymerization of ε-caprolactone to prepare *di*-blcok poly(HB-*co*-CL)s, or ring-opening polymerization of trimethylene carbonate to prepare *di*-blcok poly(HB-*co*-TMC)s.

3. New enzymatic method will be established to prepare block copolymers *via* polycondensation. The polycondensation of telechelic PHB-diol and PHO-diol *via* one-step or two-step enzymatic synthesis will lead to the preparation of copolyesters with randomly arranged or A-B type multi-block copolymers.

Therefore, this thesis will focus on the preparation and characterization of microbial PHB-based copolymers as PHB is a prominent natural polymer with good biocompatibility and thermoplastic properties. Novel methods for prepare PHB-based block copolymer with well-defined structures will be established. The optimized reaction conditions of preparing PHB-based block copolymers will be systematically investigated. Different block copolymers prepared from different combination of monomers will be examined. The effect of polymer chain length and concentration of hard domain and soft domain on the physical and mechanical properties will be studied. The biocompatibility of the prepared block copolymers will be confirmed by *in vitro* cell culture in future. The possible application of the block copolymers as biomaterials in biomedical fields would be of great interest for further study.

1.4 Outline of this thesis

Different methods of the modification of PHB to achieve improved physical and mechanical properties were reviewed in chapter 2. The synthesis routes of preparing PHB-based random or block copolymers by different catalysts were compared. The possible polyesters / polycarbonates as candidates for biomaterials synthesis by enzymatic polymeriszaion were also discussion.

In chapter 3, an enzymatic synthesis route of preparing block copolyester was established via the ring opening polymerization of cycilic lactone (ε-caprolactone) by using telechilic macro-diol as initiator. The optimization of reaction conditions was investigated and the analytical methods for both structure and physical properties analysis were established. Di-block poly(HB-co-CL)s with different molecular weight and physical properties were synthesized with well defined structure.

To expand the application of the mentioned method, more synthetic work was performed in chapter 4. The chemo-enzymatic polymerizations of poly(HB-co-TMC)s and poly(TMC-co-CL-co-TMC)s was studied via the ring opening polymerization of trimethylene carbonate with differnet telechelic diols as initiator. The prepared block copolymers were also used as macro-diols for polyurethanes synthesis to achieve better mechanical properties.

Another green and efficient method of polycondensation of macrodiols with divinyl adipage was also established in chapter 5. Block copolyesters of poly(HB-co-HO)s were prepared from natrual-origined PHB and PHO for the first time with pure PHB and PHO segements, no additional juntion units were involved in the polymer chain. By one-step synthesis and two-step synthesis, the arrangment of PHB and PHO block in poly(HB-co-HO)s were well controlled, and the physical properties were improved accordingly.

The conclusions from the research work in chapter 3-5 were summarized and emphasized in chapter 6. The possible research interests in this area were also proposed in chapter 6.

CHAPTER 2

LITERATURE REVIEW

2.1 Modification of PHB

Microbial produced PHB is a thermoplastic material, thus being melt-processable. It can be injection-moulded beyond its melting temperature, but it is also melt unstable because the degradation temperature is relatively close to its melting temperature. PHB may also be degradable to crotonic acid if kept for a relatively long time at a temperature of only a few degrees above its melting point. Injection-moulded PHB bars show high crystallinity with brittle behavior, especially at the temperatures below its glass transition temperature.[22] Thus, the narrow processability window and relatively low impact resistance were found to be significant limitations for the direct applications of PHB. Different methods have been explored to improve the properties of PHB. These efforts may be classified into four categories: physical modification, biological modification, chemical modification as well as enzymatic modification of PHB.

2.1.1 Physical Modification of PHB

The properties of PHB are mainly governed by its natural chemical structure, such as the bonding energy. However, the extrinsic factors may also play an important role. For instance, the morphology of PHB granules or the cracks in the micro-structure of PHB may also determine the physical or mechanical properties of PHB. Physical modification of PHB mainly aimed at the improvement of its micro-structure by annealing or the modification of its morphology by forming blends with other materials.

2.1.1.1 Annealing

The improvement of properties of PHB by annealing had been studied since 1980's. Barham and Keller reported the first example that annealing can be applied to modify the properties of PHB. [23] They found the brittleness of PHB was due to the cracks within PHB, which may be either radially or circumferentially distributed. When the PHB granules were strained, the cracks may grow with the additional force, and then resulted in brittle failure. They tried to heal these cracks inside the PHB granules by rolling the films to decrease the brittleness. In 1993, de Koning reported that the chain scission physical aging did not contribute to the embrittlement of PHB at ambient temperature, while the progressive crystallization was the main reason of the brittle failure, and this may be significantly prevented to by simple annealing,[24] since annealing appeared to induce structure reorganization by melting and re-crystallization. By optimizing the annealing temperature and time, the extension at break of PHB can be achieved as 30% when it was annealed at 150 ^{o}C.[25] Hobbs and coworkers also sudied the effect of annealing temperature on the mechanical properties of PHB. Annealing at a temperature of 120 ^{o}C or above was found to result in increases in both critical strain energy release rate and stress intensity factor at fracture. When the annealing temperature was raised to 130 ^{o}C, PHB was found to be a material with significantly improved fracture properties, which were to a large extent maintained on subsequent re-aging.[26] This method provided with a simple route to improve the properties of PHB by controlling and modifying its micro-structure. Furthermore, it was applied to achieve better mechanical and thermal

properties of PHB fibers[27] or PHB-based copolymers, such as poly(3HB-*co*-3HV)s and poly(3HB-*co*-4HB)s.[28]

2.1.1.2 Blending

Another physical method to modify the properties of PHB is to form PHB-based polymer blends. A polymer blend, polymer alloy, or polymer mixture is a member of a class of materials analogous to metal alloys, in which two or more polymers are blended together to create a new material with different physical properties.[29] Different from annealing, a PHB-based polymer blend was designed to modify the morphology of PHB chains by introducing other components. Many components have been investigated to mix with PHB. In 1988, Avella *et al.* reported the thermal and crystallization behavior of a binary blend by mixing PHB and poly(ethylene oxide) (PEO). With different PHB / PEO ratio ranged from 100 / 0 to 20 / 80, the crystallization temperature (T_c) of the blends was changed from 90-140 ^oC to 90-120 ^oC, the T_m of PHB decreased from 194^oC to 163^oC, and the T_g from 9^oC to -43^oC with 80% PEO involved. Avella *et al.* also found that both PHB and PEO are crystallizable, thus the blend demonstrated a single glass transition temperature and a depression of the equilibrium melting temperature as well as of the radial growth rate of PHB spherulites. The specific interactions responsible for the PHB-PEO miscibility were likely to involve the carbonyl groups of the PHB and the hydrogen of the CH_2 group from PEO.[30]

Different from Avella's work, Greco *et al.* studied the blend by mixing PHB with un-crystallized rubbery components: ethylene-propylene rubber (EPR) and poly(vinyl

acetate) (PVAc).[31] The effects of molecular structure and characteristics of an un-crystallized rubbery component were significant on the melt miscibility, phase structure, morphology, thermal and crystallization behavior of these PHB-based blends. PHB and EPR are immiscible, while PHB and PVAc are compatible, thus PHB-PVAc blends showed a single glass transition and a drastic depression of equilibrium melting temperature of PHB. Another *un*-crystallized rubbery component, namely atactic poly(oxy-2-chloromethyl-ethylene) (PECH), was also investigated to form a blend with PHB.[32, 33] The PHB / PECH blend was confirmed to be miscible, and the influence of PECH on the spherulitic growth rate and the overall crystallization rate were explored. The PECH molecules were assumed to be rejected in the inter-lamellar or inter-fibrillar regions of PHB spherulites, where they formed a homogeneous mixture with un-crystallized PHB molecules.

Other amorphous blends were obtained by mixing PHB with cellulose acetate propionate (CAP) or cellulose acetate butyrate (CAB).[34] When the CAP or CAB component exceeds 50%, crystallization of PHB was effectively prevented and fully amorphous PHB-based blends may be obtained. With the increased interests focused on PHB and its applications, more examples of PHB-based blends were examined to achieve better properties. In 1995, Siciliano reported the miscibility and thermal behaviors of PHB and atactic poly(methyl methacrylate) (PHB/aPMMA) blend.[35] The melting temperature of the bend was found to have a close correspondence to the aPMMA content: the T_m of PHB was measured as 172 ^oC, while that of 40/60 PHB / aPMMA quenched blend was 167 ^oC. Similar studies on PHB / aPMMA blends were also investigated by Canetti[36] and Cimmino.[37] Xing *et al.* reported the blends of PHB /

poly(*p*-vinylphenol) (PVPh)[38] and PHB / Poly(vinyl acetate-co-vinyl alcohol) (PVAc-*co*-PVA) blends.[39] The PHB / PVPh blends showed a change in T_m from 186 to 171 ^oC with 0-30% PVPh incorporated, but the T_g got an obvious increase from 4.5 to 32.7 ^oC. Regarding the PHB / PVAc-*co*-PVA blends, there were quite different phase behaviors with different PVA concentrations (9% - 22%) in PVAc-*co*-PVA component.

As mentioned above, PHB is miscible with PEO, PVAc, PECH, CAP / CAB, aPMMA, PVPh and PVAc-*co*-PVA. However, the biocompatibility of these components are rather limited and only PEO is relatively biodegradable. To satisfy the requirements of biomaterials, several biocompatible and biodegradable components, such as poly(L-lactic acid) (PLLA),[40, 41] attracted great interest to form PHB-based blends. The thermal and mechanical properties of PHB were effectively modified by incorporating 10-70 wt% PLLA with PHB: the T_g of PHB / PLLA (70 wt%) was measured as -1.7 ^oC while that of the original PHB is 0.7 ^oC; the Young's modulus and tensile strength of PHB / PLLA blends were modified from 0.65 GPa to 1.8 GPa and 25 MPa to 160 MPa, respectively; the elongation at break (ε_b) was also significantly improved from 10% to 150% at different draw ratio. Furthermore, the blends of isotactic PHB with synthesized atactic PHB were also investigated by Pearce *et al.* [42-44] and Abe *et al.*.[45] The effect of tacticity of the blends on their melting behavior was systematically explored. From the DSC analysis of isotactic PHB / synthetic atactic PHB or isotactic PHB / partially isotactic PHB blends, there was a slight decrease in T_m with atactic PHB content of about 60 wt%, followed by a more dramatic decrease in T_m when atactic PHB composition above 60 wt%.[42] This blends system provided a simple and efficient approach to lower the melting temperature of the bacterial polymer without compromising its environmental qualities.

Physical modification of PHB by either annealing or blending achieved definite improvement of the thermal and mechanical properties of PHB. These physical modifications were confirmed as effective methods to improve the micro-structure of PHB, thus successfully controlled its crystallization behavior. Some of those products have been launched for biomedical or agriculture applications. However, the improvement from the limited changing range of bonding force was still insufficient for specific applications.

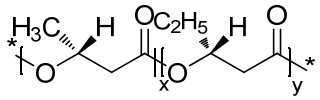
2.1.2 Biological Modification of PHB

Biosynthesis of co-polyesters containing PHB and other PHAs units is another efficient approach to overcome the shortcomings of PHB, and to obtain useful PHB-based new materials. Biological modification of PHB developed the fermentation procedure by using specific additives in the growth medium of the bacteria, thus, new PHB-based copolymers consisting of two or more components were produced. The incorporated new monomer units in the backbone of PHB played an important role in improving the thermal and mechanical properties by the newly formed chemical bonds. The fermentation production of PHB-based copolymers is essentially similar to that of PHB homo-polymer except that a mixture of carbon source was applied. The first PHAs co-polyester was isolated from environmental samples in 1974 by Wallen and Rohwedder, and the polymer contained four different monomeric units: 3-hydroxybutyrate, 3-hydroxyvalerate, 3-hydroxycaproate, and 3-hydroxyheptanoate.[46] From that on, different PHAs copolymers have been isolated from different environmental samples, including

marine sediments,[47] marine and freshwater cyanobacteria,[48] and sewage sludges.[49] As an successful example, poly(3-Hydroxybutyrate-*co*-3-Hydroxyvalerate) (PHBV) has been known as the most prominent PHA-based copolymer, and been commercially produced in large-scale.

2.1.2.1 Poly(3-Hydroxybutyrate-*co*-3-Hydroxyvalerate)

In 1981 Holemes from ICI developed a controlled-fermentaion process to produce poly(3-Hydroxybutyrate-*co*-3-Hydroxyvalerate) (PHBV) by *Alcaligenes eutrophus* grown in a culture medium containing glucose and propionate.[50-52] The composition of PHBV has been reported to vary from 0-47 mol% P3HV, depending on the composition



Scheme 2.1 Structure of Poly(3HB-co-3HV)

of the feeding carbon substrates.[53] The Young's modulus, tensile strength and the melting temperature have been shown to be regulated by the content of HV units in the co-polyester.[54] By using different carbon sources, PHBV with a much wider range of compositions can be produced. For example, PHBV was found to be accumulated in *Alcaligenes eutrophus* cells in a nitrogen-free culture solution containing acetic and propionic acid with HV fraction up to 44 mol%,[52] while the HV content can reach up to 95 mol% by using pentanoic and butyric acid as the carbon souces in *Alcaligenes eutrophus*.[55, 56] The thermal properties and mechanical properties of PHB were significantly improved by incorporating 25 mol% HV units in PHBV: the T_m changed

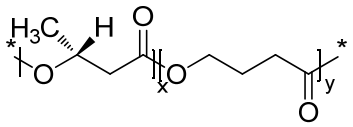
from179 ^{o}C (original PHB) to 137 ^{o}C and T_g from 10 ^{o}C to -6 ^{o}C; the Young's modulus of PHBV also got a dramatic decrease from 3.5 GPa to 0.7 GPa, while the tensile strength remained around 30 MPa.[21]

As one of the major product from microbial PHAs family, PHBV had attracted great attention of being candidate for large-scale biotechnological production, since it is environmentally degradable and thermoplastic polyester. It also has good mechanical properties, which were comparable to commercial thermoplastic polymers, such as isotactic polypropylene and poly(ethylene terephthalate), in 1980's. The commercialized microbial PHBV copolymer (Biopol®) production was first launched by ICI,[14] and they also set up Marlborough Biopolymer Limited (MBL) to exploit Biopol® in 1983. Nowadays, PHBV was used for clinical applications, such as sutures, drug delivery devices and so on, due to its biocompatibility and biodegradability.

2.1.2.2 Poly(3-Hydroxybutyrate-*co*-4-Hydroxybutyrate)

Poly(3-Hydroxybutyrate-*co*-4-Hydroxybutyrate) (poly(3HB-*co*-4HB)) is another important microbial produced PHA copolymer. It was firstly found by Doi, Kunioka and coworkers as a new polyester accumulated in *A. eutrophus* with 4-hydroxybutyric acid was applied as the carbon source.[57-59] Micirobial poly(3HB-*co*-4HB) has a statistically random distribution of 3HB and 4HB units, and was found to be an useful thermoplastic material. Different combinations of carbon sources have been studied for poly(3HB-*co*-4HB) production. 4-hydroxybutyric acid or γ–butyrolactone are the most prominent substrates applied as carbon source to produce poly(3HB-*co*-4HB) by *A. eutrophus*.

Other carbon sources including 4-chlorobutyric acid, 1,4-butanediol, and 1,6-hexanediol have also been reported to produce poly(3HB-*co*-4HB) by *A. eutrophus*.[59, 60]



Scheme 2.2 Structure of Poly(3HB-co-4HB)

When 4-hydroxybutyric acid was applied as the solo carbon source, the poly(3HB-*co*-4HB) copolymer composed of 64 mol% 3HB and 36 mol% 4HB was produced under the nitrogen-free culture media with 4-hydroxybutyric acid concentration of 28 g/L. Carbon source concentration was also found to have significant effect on the composition of poly(3HB-*co*-4HB). The fraction of 4HB increased from 25 mol% to 36 mol% with the increasing of carbon source concentration from 4 g/L to 28 g/L.[1] The addition of butyric acid to the 4-hydroxybutyric acid culture solution resulted in a decrease in the 4HB fraction of the copolymer. Thus, the copolymer compositions vary from 0–36 mol% 4HB depending on the different carbon sources compositions.

The physical properties of poly(3HB-*co*-4HB) with 0-18 mol% 4HB in powder form were analyzed by Kunioka *et al.* in 1989. The T_m of poly(3HB-*co*-4HB) decreased from 178 ^oC to 150 ^oC when 4HB concent increased from 0 to 18 mol%.[61] The T_g of poly(3HB-*co*-4HB) (0-82 mol% 4HB) showed a significant decrease from 4 ^oC to -41 ^oC as 4HB content increased from 0 to 82 mol%.[62] Similar results were also reported by Saito *et al.*, the T_g of poly(3HB-*co*-4HB) decreased from 4 ^oC to -48 ^oC as the 4HB content increased from 0 to 100 mol%.[63, 64] The 4HB units were relatively rubbery than 3HB units. The producing of P4HB units during the cell growth significantly limited the

crystallization behavior of 3HB units, thus demonstrated different thermal properties of poly(3HB-*co*-4HB).

The mechanical properties of poly(3HB-*co*-4HB) film have been studied with 0-44 mol% 4HB composition range by Doi *et al.*.[65] The crystallinity decreased gradually from 60±5% to 15±5% with 4HB content increased from 0 to 44 mol%. Under the same conditions, the tensile strength of poly(3HB-*co*-4HB) decreased from 43 MPa to 10 MPa, while the elongation at break (ε_b) was improved from 5% to 511%. Saito's work demonstrated that the tensile strength of poly(3HB-*co*-4HB) film with composition of 0-16 mol% decreased from 43 MPa to 26 MPa with an increase of 4HB fraction. The elongation at break increased from 5 to 444%, and the tensile strength of the films with composition of 64 to 100 mol% 4HB increased from 17 to 104 MPa with the increase of 4HB fraction.[64] From these reported results, it can be concluded that the microbial produced poly(3HB-*co*-4HB) had improved thermal and mechanical properties compared with PHB homo-polymer. The incorporation of 4HB units effectively provided desirable contribution to achieve improved properties of PHB.

2.1.2.3 Other PHA copolyesters

Besides PHBV and poly(3HB-*co*-4HB), there were other co-polyesters successfully prepared by using specific additives in growth medium. Shimamura *et al.* (1994) reported the microbial synthesis of poly[3HB-*co*-3HP(hydroxypropionate)] by a single stage fermentation of *A. latus* using hydroxybutyric acid (HBA) and hydroxypropionic acid (HPA) as carbon sources.[66]

(i) (ii)

Scheme 2.3 Structure of Poly(3HB-co-3HP) (i) and Poly(3HB-co-3HHx) (ii)

Poly(3HB-*co*-3HP)s were produced with 3HP content up to 88 mol%. The T_m of poly(3HB-*co*-3HP) with different 3HP content (0-88 mol%) ranged from 177 ^oC to 44 ^oC and T_g of 4 ^oC to -15 ^oC. The crystallinity of poly(HB-*co*-HP) was clearly decreased from 60±5% to 13±5% with the 3HP units concentration increased from 0 mol% to 71 mol%.

Doi (1995) demonstrated the microbial synthesis of poly(3HB-*co*-3HHx)s by a two-stage batch fermentation of *A. caviae* 440 using alkanoic acids (C11-C18) as carbon sources.[67] The composition of co-polyesters was determined by integration of the proton resonances of 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 3-hydroxyhexanoate (3HHx) units by ^1H NMR analysis. Poly(3HB-*co*-3HHx)s were prepared with 3HHx content of 0-25 mol%, and the corresponding T_m of 177 ^oC to 44 ^oC and T_g of 4 ^oC to -4 ^oC. Furthermore, multiple-components co-polyesters were studied by Abe *et al.* (1994).[68] The co-polyesters consisting of 3HB and medium-chain-length 3HA units of even carbon number (C6, C8, C10 and C12) by *Pseudomonas sp.* 61-3 were synthesized from gluconate, and all these materials showed better flexibility than that of original PHB.

Biological modification indicated efficient improvements of thermal and mechanical properties of PHB by forming microbial PHB-based copolymers. However, the additives used in the fermentation are expensive, and production yields are considerably lower than for PHB homo-polymer owing to their toxicity in the culture.

The mechanical and thermal properties improvements are at the expense of both production and processing costs. Even so, the copolymers constitute the present commercial production of Biopol ® because of their superior mechanical behaviors. Although the biological modification of PHB was still limited by the available carbon sources, the developed genetic technology may help to produce better bacteria-based plastic as desired components in PHB-based copolymers.

2.1.3 Chemical Modification of PHB

To overcome the limitation of co-monomers for microbial synthesis of PHB-based copolymers, chemical synthetic route provides more possibilities to produce PHB-based copolymers which can not be produced through biological way. PHB-based copolymers can be produced either by ring-opening polymerization of two or more monomers including *β*-butyrolactone to form random copolymers, or by applying PHB as a whole block to synthesize new block copolymers. Both random copolymers and block copolymers can effectively improve the mechanical and thermal properties of PHB by forming new chemical bonds on the original PHB backbone.

2.1.3.1 PHB-based Random Copolymer *via* Ring-Opening Polymerization

Chemical ring-opening polymerization of *β*-butyrolactone (BL) with other co-monomers was an attractive method to synthesize PHB-based random copolymers. Many catalysts, such as tin(IV) complexes,[69-71] yttrium complexes,[72] zinc complexes,[73, 74]

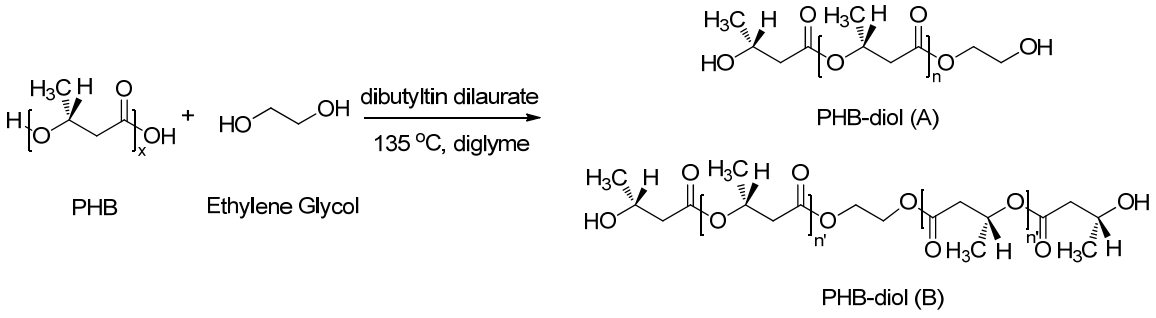
aluminum complexes,[75, 76] and dibutylmagnesium,[77] have been investigated for the ring-opening polymerization of *β*-butyrolactone. The catalysts containing different catalytic centers showed efficient activity for the preparation of PHB from cyclic monomer. The application of those catalysts was extended for the ring-opening polymerization of other cyclic monomers. The ring-opening polymerization of *β*-butyrolactone with other cyclic monomers has also been studied. Benvenuti *et al.* synthesized random copolymer of poly(*β*-butyrolactone-*co*-*β*-benzyl malate) [Poly(HB-*co*-BM)] *via* ring-opening polymerization of racemic *β*-butyrolactone and racemic *β*-benzyl malolactonate using ethylaluminoxane (EAO) as catalyst. The molecular weight of poly(HB-*co*-BM) was up to 77,000 Da in about 75 % yield after 5 to 10 days' reaction.[78] Jaïmes, Schué and Arcana focused their interests on the preparation of poly(HB-*co*-CL)s and poly(HB-*co*-HV)s *via* ring-opening polymerization of *β*-butyrolactone with *ε*-caprolactone or *β*-valerolactone using tetraisobutyldialuminoxane (TIBAO: $(iBu)_4Al_2O$) or Bu_2SnO / Bu_2SnCl_2 as catalyst.[76, 79, 80] Poly(HB-*co*-CL)s with CL content of 8-95 % showed a T_g of -3 to -54 ^oC in a M_n range from 5,600 to 19,800 Da, while poly(HB-*co*-VL)s with VL content of 10-89 % showed a T_g of -4.5 to -54 ^oC with M_n of 2,800 to 7,650 Da.[76] Wei *et al.* (2006) reported the preparation of random copolymers of poly(3HB-*co*-4HB) and poly(3HB-*co*-3HV) *via* ring-opening polymerization of *β*-butyrolactone with *γ*-butyrolactone or *β*-valerolactone, respectively. Poly(3HB-*co*-3HV)s achieved a high yield of 96-99% with the 3HB / 3HV ratio of 0 / 100 to 87 / 13, while poly(3HB-*co*-4HB)s had a relatively lower yield of 46-95% with 3HB / 4HB ratio ranged from 72 / 28 to 96 / 4.[81]

The random copolymers prepared *via* metal-catalyzed ring-opening polymerization demonstrated improved thermal properties compared with those of PHB,

but in these random copolymers, the backbone structures have already been quite different from that of microbial produced PHB. Thus, the original biological properties of microbial PHB, such as the biocompatibility and biodegradability may also be changed. It will be helpful if the main structure of microbial PHB could be remained in the copolymers as a whole block. This idea was supported by PHB-based block copolymer synthesis by utilizing functionalized microbial PHB as starting material *via* polycondensation or ring-opening polymerization.

2.1.3.2 PHB-based Block Copolymers *via* Polycondensation or Ring-opening polymerization.

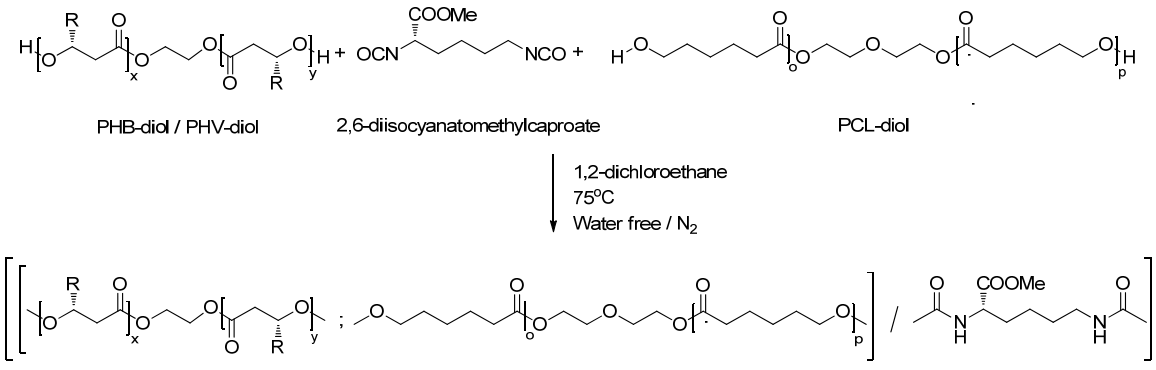
Block copolymers cover a wide spectrum of properties due to the phase segregation of their different, concatenated polymeric constituents. PHB-based block copolymers have also been widely investigated. Many soft domains, which provided lower crystallinity, lower melting temperature, and lower young's modulus, were applied to form block copolymers with functionalized PHB. Hirt *et al*. (1996) explored a method to functionalize PHB through transesterification between high molecular PHB and ethylene glycol by using dibutyltin dilaurate as catalyst in diglyme (Scheme 2.4).[82] Low-



Scheme 2.4 Transesterification of PHB and Ethylene Glycol to Produce PHB-diol

molecular-weight hydroxylated PHB (PHB-diol) was successfully produced, and was further applied for PHB-based poly(ester-urethane) synthesis.[83]

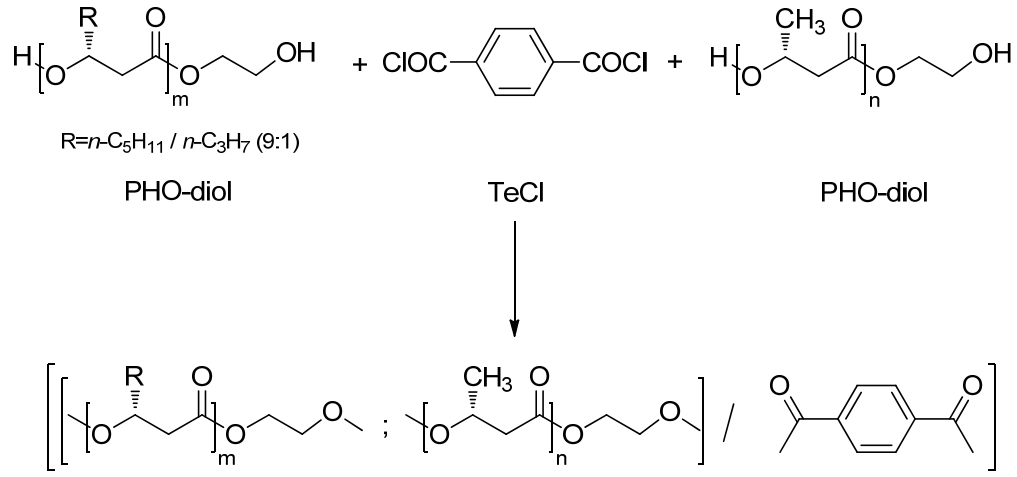
With this functionalized PHB as starting material, the preparation methods of PHB-based block copolymers were generally classified into two categories: the poly(ester-urethane)s or polyesters prepared *via* polycondensation from PHB-diol with other diols of polyesters linked by different junction units, and the polyesters or polyethers prepared *via* ring-opening polymerization of other cyclic monomers with PHB-diol as macro-initiator. Different soft domains, such as *α,ω*-dihydroxy-[poly(*ε*-caprolactone)-*co*-(diethylene glycol)-*co*-poly(*ε*-caprolactone)] (PCL-diol) and *α,ω*-dihydroxy-[poly(adipic acid)-*alt*-poly(1,4-butanediol; diethylene glycol; ethylene glycol)] (Diorez®), have been investigated for PHB-based block copolymers synthesis with PHB-diol *via* polycondensation by using L-lysine methyl ester diisocyanate (LDI) or 2,2,4-trimethylhexamethylene diisocyanate (TMDI) as junction units (Scheme 2.5).[83]



Scheme 2.5 Polyurethan Prepared by Chain Extension with LDI of PHB/HV-diol and PCL-diol

The obtained poly(ester-urethanes)s showed obvious change in crystallinity and T_m with different PHB content: the crystallinity ranged from 20% to 41%, and T_m ranged from 112 °C to 134 °C. Both crystallinity and T_m got significant decrease compared with that of

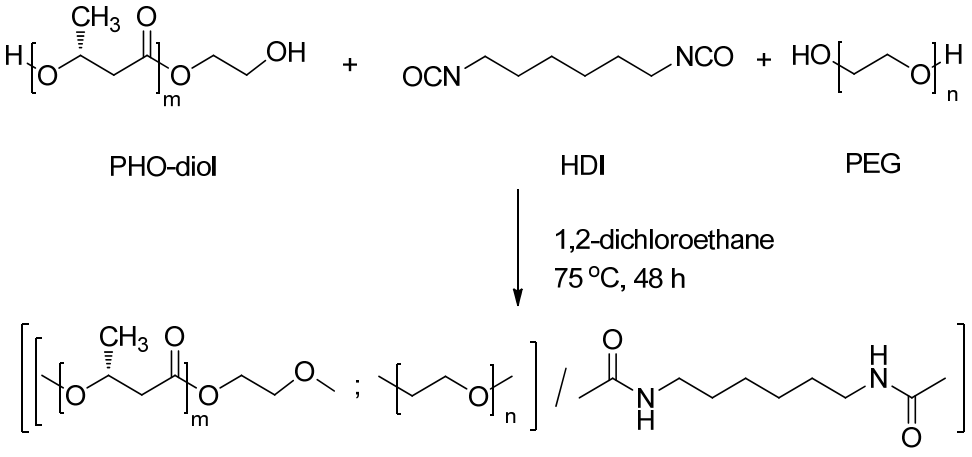
PHB. Although the thermal and mechanical properties of PHB were well modified by forming poly(ester-urethane)s copolymers, junction units, such as LDI and TMDI, were applied in these reactions. As the results, the -NCO- groups were involved in the obtained copolymers, and thus decreased the biodegradability of the materials. Moreover, the residual junction units may also result in high toxicity of the copolymers. Andrade et al. (2002) reported the poly(ester-urethane) containing PHB as hard domain and poly-(hydroxyoctanoate) (PHO) as soft domain.[84] Compared with original PHB-diol, the T_m of poly(HB-*co*-HO)s decreased from 177°C to 146°C and the T_g changed from 4°C to -6°C. In these copolymers, junction unit of LDI was also involved. With another junction unit of terephthaloyl chloride (TeCl), a block co-polyester containing PHB and PHO block was prepared with T_m and T_g of 140°C and -41°C were obtained respectively (Scheme 2.6).[85]



Scheme 2.6 Polyester Prepared by Chain Extension with TeCl of PHO-diol and PHB-diol

Poly(ethylene glycol) (PEG) was also reported be applied as soft segment in modification of PHB. PEG is a highly hydrophilic synthetic polyether, and it also has good biocompatibility and flexibility. Similar to the poly(ester-urethane)s preparation,

PHB / PEG block copolymers can also be easily obtained through one-step condensation polymerization of PHB-diol and PEG with diisocyanate as coupling agent. Zhao *et al.* (2004) reported the synthesis of multi-block copolymers containing PHB and PEG blocks. PHB-diol (M_n of ~3000) and PEG (M_n of 1000, 4000, and 6000) were applied for the polycondensation with 1,6-hexamethylene dissocyanate (HDI) as junction unit.[86] With the same synthetic strategy, *tri*-block poly(ester-urethane)s of PEG-PHB-PEG were also reported by Li *et al.* (Scheme 2.7).[87] The prepared *tri*-block copolymers with PEG content of 54.3% to 87.3% showed quite different thermal and mechanical properties from those of original PHB. The T_m from PHB block was measured as 82.2 ^oC with 80% PEG incorporated in the copolymer, it was much lower than that of PHB-diol (135.0 ^oC). The T_m from PEG block also decreased from 61.6 ^oC to 40.9 ^oC. The Young's modulus of PHB (1143±72 MPa) was modified to a lower lever of 21±1 MPa with PEG content of 61.6% in the copolymer. The best elongation at break was achieved as 1912±120 % with the PEG content of 87.3%.



Scheme 2.7 Polyurethan Prepared by Chain Extension with HDI of PHB-diol and PEG

Different from the poly(ester-urethane)s synthesis, the *tri*-block copolymer of PEO-PHB-PEO was reported by chemical method without involving any junction unit.[88] Low-molecular-weight PHB-diol was prepared according Hirt's method[82] and PEO was functionalized as methoxy-PEO-monocarboxylic acid (M-PEO-A), then PHB-diol were allowed to react with M-PEO-A catalyzed by 1,3-N,N'-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamion)pyridine (DMAP). This reaction gave *tri*-block copolymers of PEO-PHB-PEO with PEO block content of 41.0% to 92.4% under various reaction conditions. By incorporating PEO block, the T_m of PHB block and PEO block were modified from 155.2 ^oC to 140.2 ^oC and 53.2 ^oC to 23.3 ^oC, respectively.

From all the mentioned PHB-based copolymers, the starting material of PHB-diol was prepared from natural biopolymer with good biocompatibility. However, another synthetic strategy for PHB-based block copolymers was developed *via* ring-opening polymerization of *β*-butyrolactone with macro-initiator. The synthetic PHB block in these copolymers are quite different from the microbial PHB, thus the biocompatibility would be also different. Liu *et al.* (2008) studied the controlled synthesis of amphiphilic PHB-PEG-PHB *tri*-block copolymer *via* ring-opening polymerization of racimic *β*-butyrolactone with PEG as macro-initiator.[89] Furthermore, PHB-based copolymer can also be prepared by a two-step ring-opening polymerization of *β*-butyrolactone and *ε*-caprolactone.[90] Abe *et al.* (1994) reported the ring-opening polymerization of *β*-butyrolactone, then the prepared PHB was further used as macro-initiator for the ring-opening polymerization of *ε*-caprolactone, thus the block copolymer of poly(HB-co-CL) were prepared.

PHB-based block copolymers prepared by chemical method were designed with well-defined backbone structure. Generally, PHB was applied as hard domain and another relatively rubbery or semi-crystalline polymer as soft domain. The properties of these block copolymers can be easily controlled by varying the ratio between PHB block and the soft domain. All of these reactions were catalyzed by chemical catalyst, such as dibutyltin dilaurate, in which heavy metal atoms were involved. The heavy metal atoms normally can not be completely removed after the reactions, and the residual catalyst will increase the toxicity of the polymers. Thus, the produced materials may have low biocompatiblitiy and limited applications in biomedical fields.

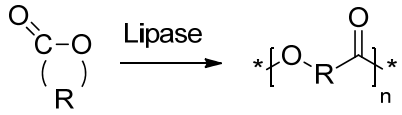
2.2. Enzyme as Catalyst in Polymer Synthesis

Enzymes are biomolecules that can catalyze chemical reactions.[91, 92] They have attracted great intention of being applied in *in vitro* organic reactions. Many types of enzymes have been explored to be useful catalyses that can produce a wide range of compounds with specific applications.[93-97] Different from chemical catalyses and designed by nature, enzyme-catalyzed reactions are normally proceed under mild reaction conditions with remarkable catalytic efficiency at different temperatures, pressures, and pH values. *In vitro* enzyme-catalyzed reactions are generally performed in bulk or organic media. Due to their high chemo-, regio- and stereo-selectivity, enzymes have been widely applied as versatile synthetic tools in pharmaceutical and fine chemical syntheses. Although enzymes may exhibit different selectivity and decreased activity in organic solvent, they can catalyze some reactions, which are impossible to carry out

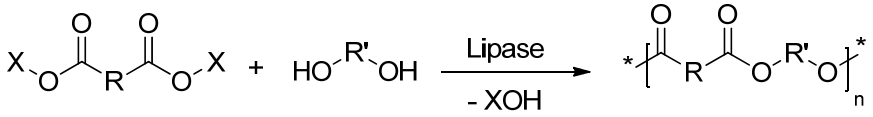
otherwise.[98] The most attractive point of enzyme catalysts for applications is the *non*-toxicity, which make enzymes as "green" catalysts.

As we know, production of all naturally occurring polymers is *in vivo* catalyzed by enzymes. Thus, the *in vitro* synthesis of polymers through enzyme catalysis has also been of great research interests and been extensively studied.[99-103] Enzymatic polymerization was defined as chemical polymer synthesis *in vitro via* non-biosynthetic pathways catalyzed by an isolated enzyme.[104] Many families of enzymes, including oxidoreductases, transferases, hydrolases, have been applied for polymer syntheses. With the great developments in biomaterial fields, enzyme-catalyzed preparation of polyesters for biomedical applications has been intensively investigated. Lipase-catalyzed syntheses of polyesters or polyester-carbonates were the most common examples.

Ring-Opening Polymerization of Cyclic Lactones:

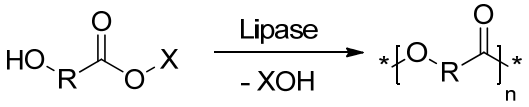


Polycondensation of Dicarboxylic Acids or their Derivatives with Glycols:



X= H, Alkyl, Halogenated Alkyl, Vinyl, etc.

Polycondensation of Oxyacids or their Esters:



X= H, Alkyl, Halogenated Alkyl, Vinyl, etc.

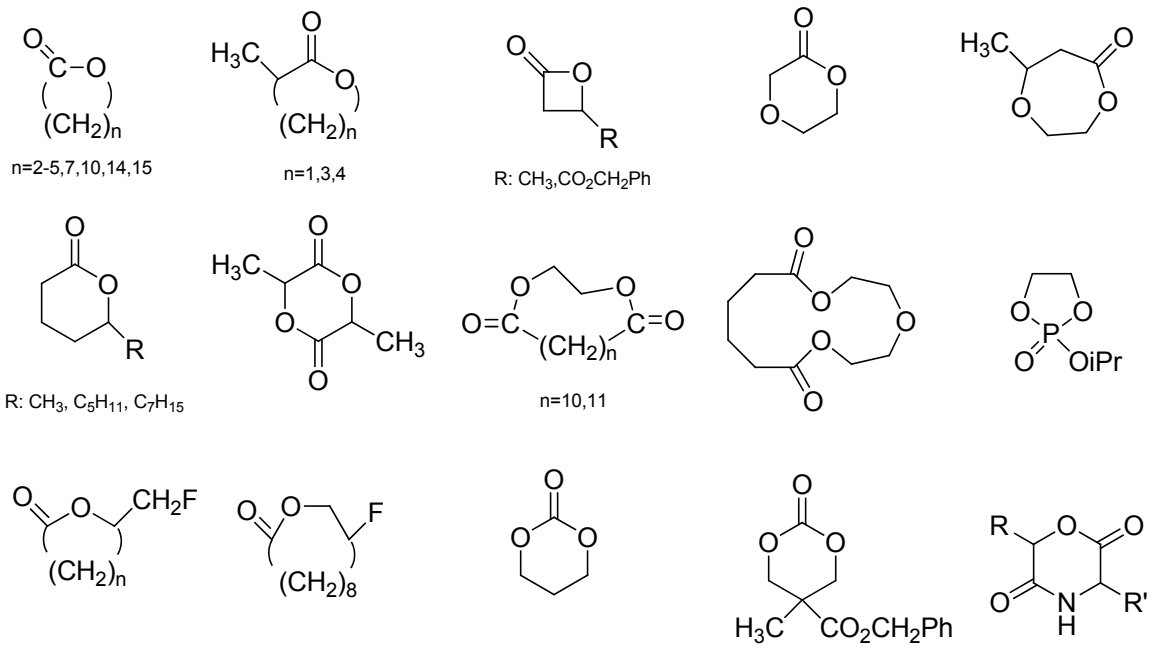
Scheme 2.8 Enzyme-Catalyzed Polymerization Reactions

Lipase is a water-soluble enzyme that can catalyze the hydrolysis of fatty acid esters. Some lipases are stable in organic solvents and can be applied in esterification and transesterifications.[105-110] Different types of lipases catalyzed different kinds of polymerizations and thus produce useful polyesters or polycarbonates.[111] Typical lipase-catalyzed ring-opening polymerization and polycondensation leading to polyesters or polycarbonates are summarized in the following sections (Scheme 2.8).[104]

2.2.1 Lipase-catalyzed Ring-Opening Polymerization of Cyclic Monomers

The first example of lipase-catalyzed ring-opening polymerization reaction was reported by Knani *et al.* and Uyama *et al.* in 1993. Knani *et al.* reported the ring-opening polymerization of *ε*-caprolactone by using crude *Porcine pancreatic lipase* (PPL) in *n*-hexane.[112] PCL with molecular weight of 613 to 1924 was obtained in 62.0 to 98.5% yield after a long reaction time of 4 to 26 days at 40 ^{o}C. Uyama *et. al.* explored the ring-opening polymerization of *ε*-caprolactone and *δ*-valerolactone by using different lipases derived from *Pseudomonas fluorescens* (Lipase PF), *Candida cylindracea* (Lipase B), and *Porcine pancreas* (PPL), respectively.[113] The polymerizations were carried out in bulk for 10 days, PCL with M_n of 1100 to 7700 in 8-92% monomer conversion and PHV with M_n of 1600 and 1900 in 95% monomer conversion were achieved. The polymerization of *ε*-caprolactone at 75 ^{o}C gave the highest monomer conversion and polymer molecular weight. Thereafter, lipase-catalyzed ring-opening polymerization of various substrates with cyclic structure, including cyclic lactones and carbonates, have

been extensively studied. The structures of some representative cyclic monomers were summarized in Scheme 2.9.[104]



Scheme 2.9 Cyclic Monomers for Lipase-Catalyzed Ring-Opening Polymerization

Lipase-Catalyzed Ring-Opening Polymerization of non-Substituted Cyclic Lactones:

Lipase-catalyzed ring-opening polymerization of cyclic lactones leads to the "Green" preparation of polyesters, which are very important materials for biomedical applications. The cyclic lactones with a ring size ranged from 4 to 17 members (*β*-propiolactone (4-membered),[114-117] *β*-butyrolactone (4-membered),[116-119] *γ*-butyrolactone (5-membered),[116, 117, 119] *δ*-valerolactone (6-membered),[113, 117, 120] *ε*-caprolactone (7-membered),[116, 117, 120-126] 8-octanolide (9-membered),[127, 128] 11-undecanolide (12-membered, UDL),[117, 129] 12-dodecanolide (13-membered, DDL),[117, 130] 15-

pentadecanolide (16-membered, PDL),[129, 131-133] and 16-hexadecanolide (17-membered)[134]) have been extensively investigated to produce the corresponding polyesters catalyzed by different types of lipases, which include crude or immobilized lipases of *Porcine pancreas* lipase (PPL), *Candida rugosa* lipase (Lipase CR), *Candida antarctica* lipase (Lipase CA), *Candida cylindracea* lipase (Lipase CC), *Candida antarctica* lipase B (CALB), *Pseudomonas cepacia* Lipase (Lipase PC), *Pseudomonas fluorescens* Lipase (Lipase PF) and so on. Crude lipases can be directly applied for the ring-opening polymerization, but there is always a high amount of lipase will be needed due to the deactivate effect of organic solvents. On the other hand, immobilized lipases provided with better stability than crude lipases, and thus have relatively higher catalytic activity for the polymerization. Immobilized lipases can also be easily recycled, and the lower production costs became a big advantage over crude lipases.

Effect of Ring Size of Cyclic Lactones

There are lots of factors affect polymerization degree and monomer conversion of lipase-catalyzed ring-opening polymerization of cyclic lactones. The effect of monomer ring size on the polymerization has been intensively studied. Due to the slow propagation kinetics, the reported lipase-catalyzed ring-opening polymerization of medium size (4-7 membered ring) cyclic lactones synthesized the corresponding polyesters with relatively low molecular weight ranged from several hundreds to 12 thousands, while the molecular weight of 11-17 membered cyclic lactones can reach up to 86 thousands.[133] The significant effect of ring size on polymerization degree was studied by Nobes *et. at.*

(1996)[116] and Kobayashi *et. al.* (1998).[135] Nobes *et. al.* examined PPL or lipase PC catalyzed ring-opening polymerization of *β*-propiolactone, *β*-butyrolactone, *γ*-butyrolactone and *ε*-caprolactone. They found that the ring strain of these different lactones had no significant effect on the rate or polymerization degree, which indicated that ring-opening step was not the control step in the polymerization. Kobayashi *et. al.* explored the relationship between $V_{max(lactone)}$ / $K_{m(lactone)}$ and polymerization degree by the ring-opening polymerization of *δ*-valerolactone, *ε*-caprolactone, UDL, DDL and PDL. They found that the $V_{max(lactone)}$ / $K_{m(lactone)}$ values of the macrolides (UDL, DDL and PDL) were larger than that of *ε*-caprolactone, and the $V_{max(lactone)}$ / $K_{m(lactone)}$ values got an increase trend with the ring size of the lactones. These results suggested that the process of lipase-lactone complex to the acyl-enzyme intermediate is the key step of the polymerization.

Lipase-Catalyzed Ring-Opening Polymerization of Substituted Cyclic Lactones:

With a chiral center available in the substituted cyclic monomers, substituted cyclic monomers may be interesting substrate for lipase-catalyzed ring-opening polymerization. Due to one of the advantage of enzyme catalyst, enantio-selectivity of lipase may result in an enantio-rich polymer with specific physical and mechanical properties. As successful examples, 4-substituted *ε*-caprolactone[136] and *α*-methyl-*β*-propiolactone (MPL)[137] have been investigated for the lipase catalyzed ring-opening polymerization. Novozym 435 catalyzed ring-opening polymerization of *ε*-caprolactone (*ε*-CL), 4-MeCL, 4-EtCL and 4-PrCL were investigated systematically. Novozym 435

showed distinct enantio-selective of different substituted cyclic monomers. *S*-enriched polymers were obtained from the polymerization of 4-MeCL and 4-EtCL, while *R*-enriched polymer was obtained from the polymerization of 4-PrCL. However, the monomer conversions of substituted monomer (4-MeCL, 65%; 4-EtCL, 57%; 4-PrCL, 21%) were rather lower than that of *non*-substituted ε-CL (>95%) under the same reaction conditions.[136] Lipase PS-30 (from *Pseudomonas fluorescens*) catalyzed ring-opening polymerization of MPL produced *S*-enriched PMPL with M_n of ca 3000 after a long reaction time of 3 to 6 days.[137] Other cyclic monomers, such as 1,4-dioxan-2-one (PDO)[138] and 3-methyl-4-oxa-6-hexanolide[135], were also studied for the lipase catalyzed ring-opening polymerization. Generally, lipase-catalyzed ring-opening polymerization of substituted monomer showed a lower polymerization degree than that of the *non*-substituted monomer with the same ring size. This phenomenon may due to the configuration hindrance, which was caused by the substituted groups, for the substrates to approach the active sites of the.

Effect of Solvent on Lipase-Catalyzed Ring-Opening Polymerization

As poly(ε-caprolactone) was an important polyester with great application potential, the lipase-catalyzed ring-opening polymerization of ε-caprolactone attracted prominent research interest, and was applied as a model reaction to study the reaction conditions and mechanisms.

Many organic solvents with various log*P* values, such as acetone (-0.24), acetonitrile (-0.33), benzene (2.15), 2-butanone (0.28), carbon tetrachloride (2.99),

chloroform (1.96), cyclohexane (3.44), cyclooctane (4.15), 1,2-dichloroethane (1.52), 1,4-dioxane (-0.42), heptane (4.00), *n*-hexane (3.50), isooctane (4.50), isopropyl ether (2.03), *tert*-butyl alcohol (0.93), *tert*-butyl methyl ether (1.16), tetrahydrofuran (0.46) and toluene (2.50), were applied for lipase-catalyzed ring-opening polymerization of ε-caprolactone.[116, 120, 123, 139-141] The results demonstrated that the polymerization in solvent with low log*P* value resulted in slower polymerization kinetics and lower molecular weight.[120, 140] On the other hand, the polymerization in solvents with relatively higher log *P* value, such as isopropyl ether, toluene, butyl ether and isooctane, showed much efficient monomer conversion and higher polymer molecular weight.[140] According to previous reports, proper selection of organic solvent may cause possible change of enzyme configuration, thus the catalytic sites may be altered or specifically fine-tuned correspondingly. The nature of organic solvent is well-known to be crucial for the maintenance of critical water content, which is necessary for enzyme catalysts. More hydrophilic solvents tend to strip the essential hydration water from the enzyme, thus distorting the catalytic conformation. In contrast, relatively hydrophobic solvents with higher log*P* value may help maintain a water layer that adheres to the surface of enzyme, thus keep the original enzyme configuration and catalytic activity.[141-143] However, when lipase-catalyzed ring-opening polymerizations of ε-caprolactone was carried out in solvents with high log*P* value, such as cyclohexane (3.44) or isooctane (4.50), ε-caprolactone is not soluble in the solvent and the reaction system was reported to have three phases: lipase, solvent and monomer.[141] The reaction efficiency may be dramatically decreased. Thus, log*P* value is not the only standard to choose suitable solvent for lipase-catalyzed ring-opening polymerization of cyclic lactones, the other

factors such as solvent geometry, dipole moments, solubility of substrates may also be considered.[144]

Effect of Water on Lipase-Catalyzed Ring-Opening Polymerization

For enzyme-catalyzed polymerization in organic media, water bound to the enzyme surface plays an important role in maintaining enzyme's conformational flexibility, and thus affects the catalytic activity of the enzyme.[145] Mei *et al.* investigated water-temperature relationships for Novyzym 435-catalyzed ring-opening polymerization of ε-caprolactone. With the increased enzyme water content from 0.6 to 1.9%, the rate of monomer conversion did not show significant difference, but the total number of polymer chains increased correspondingly, and a linear relationship was observed at a fixed reaction temperature of 60 ^{o}C. When the reaction temperature increased to 108 ^{o}C, the hydrogen-bonding interaction between water and catalytic proteins from enzyme might be changed, the "tightly bound" water may become more available or even "free". The relatively released water can be applied as initiator for the ring-opening polymerization, and increases the total number of polymer chain. However, the polymerization of ε-caprolactone carried out at 108 ^{o}C demonstrated identical chain numbers with that from the reaction at 60 ^{o}C. A conclusion was drawn that the temperature of 108 ^{o}C may not be sufficient to make the "tightly bound" water to "free water".[146]

The relationship between water and molecular weight of polymers was also explored by Dong *et al.* (1999) by ring-opening polymerization of ε-caprolactone with

PSL (Lipase from *Pseudomonas sp.*) as catalyst. An enzyme water content of 3.8% was suggested to achieve desired polymerization degree. When the enzyme water content of 16% was applied in the polymerization, high amounts of lower molecular weight would be produced, which may due to the hydrolysis caused by the excess water in the reaction system.[141] In addition, the water content effects on the polymerization were also studied through lipase PS-30 catalyzed ring-opening polymerization of 15-pentadecanolide,[132] and lipase PF catalyzed ring-opening polymerization of 11-undecanolide.[147] A higher molecular weight was obtained under relatively higher enzyme water content.

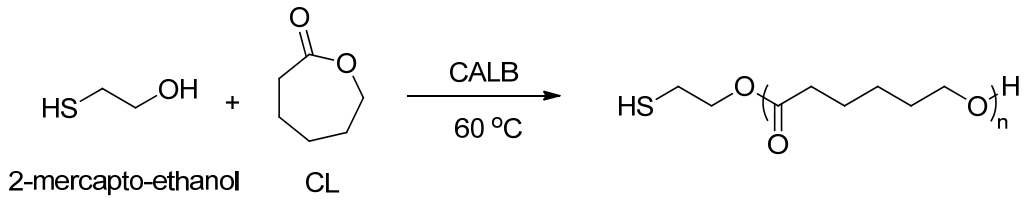
Initiators for Lipase-Catalyzed Ring-Opening Polymerization of Cyclic Lactones

To produce polyesters with functionalized ending groups, various initiators have been applied from lipase catalyzed ring-opening polymerization of cyclic lactones. Water and methanol were the most commonly applied initiators for the ring-opening polymerization of cyclic lactones to produced corresponding polyesters. One –OH end and one –COOH end will be produced when water was applied as initiator, while the methanol initiated reaction has only one functional end with –OH group.[116] Other alcohols, including 9-decenol, cinnamyl alcohol, 2-(3-hydroxyphenyl)-ethanol and 2-(4-hydroxyphenyl)-ethanol, were examined as initiator for the ring-opening polymerization of ε-caprolactone. Corresponding poly(ε-caprolactone)s were obtained with molecular weigh of *ca.* 2000.[148] Srivastava and Albertsson (2006) studied Novozym 435 catalyzed ring-opening polymerization of ε-caprolactone or 1,5-dioxepan-2-one (DXO) by using PCL-diol, 4-pentene-2-ol, or PEG as initiator, respectively.[149] 4-pentene-2-ol initiated

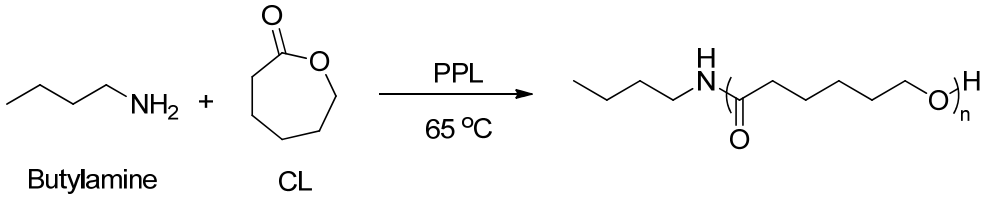
ring-opening polymerization of DXO or ε-caprolactone gave PDXO or PCL with molecular weight of 3000 to 10000 and an unsaturated terminal group was successfully introduced.

Besides the small molecules of different alcohols, oligomers with –OH or other functional ending groups may also be applied as macro-initiator for lipase-catalyzed ring-opening polymerization of cyclic monomers. PCL-diol (M_n=1250 or 2000) and PEG (M_n=1000 or 2000) were used as macro-initiator for the ring-opening polymerization of DXO or ε-caprolactone to produce *tri*-block copolymers of poly(DXO-*b*-PEG-*b*-DXO)s and poly(DXO-*b*-PCL-*b*-DXO)s with M_n ranged from 3500 to 8300. Although the macro-initiator offered slower polymerization kinetics, relatively lower monomer conversion and polymer yield, it provided a new method of preparing block copolymers *via* ring-opening polymerization.

Hedfors *et al.* reported the synthesis of thiol end-functionalized PCL *via* CALB-catalyzed ring-opening polymerization of ε-caprolactone by using 2-mercapto-ethanol as initiator (Scheme 2.10).[150] Amines instead of alcohols can also initiate the ring-opening polymerization of cyclic monomers. Henderson *et al.* investigated the effects of initiator on polymer structure and propagation kinetics by PPL-catalyzed ring-opening polymerization of ε-caprolactone (Scheme 2.11). Butylamine, butanol and water were compared as different nucleophiles for PCL preparation. Butylamine showed much faster initiation rate than that of butanol and water, but the monomer conversion and the molecular weight were lower than that from butyl alcohol initiated polymerization under the same reaction conditions.[151]



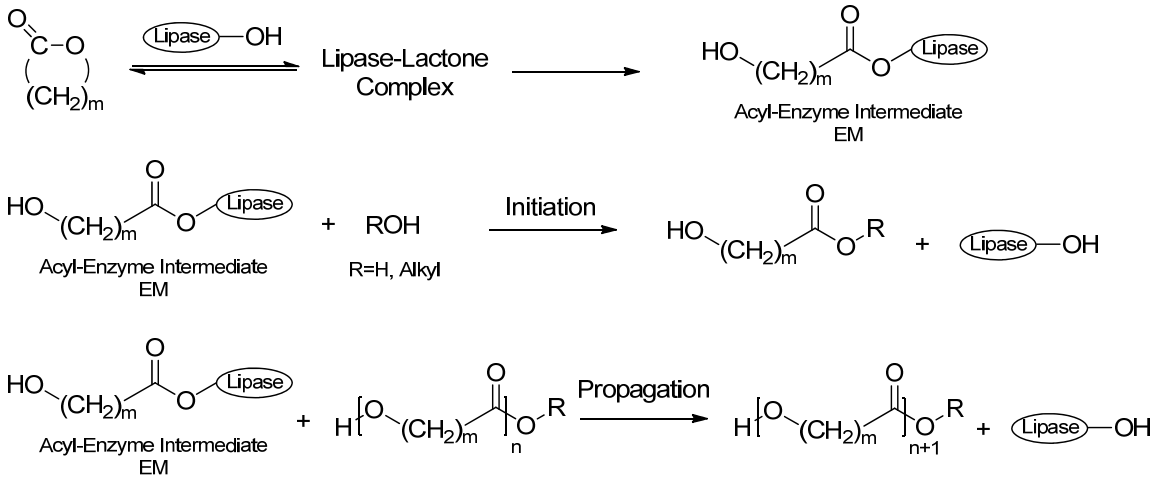
Scheme 2.10 Polymerization of Caprolactone initiated by 2-mercaptoethanol



Scheme 2.11 Polymerization of Caprolactone initiated by Butylamine

Mechanism of Lipase-Catalyzed Ring-Opening Polymerization of Cyclic Lactones

The mechanism of lipase-catalyzed ring-opening polymerization of cyclic lactones has been well studied. The most accepted theory was that the catalytic site from lipase is a serine residue, and the ring-opening polymerization was preformed *via* an acyl-enzyme intermediate. The polymerization mechanism was summarized and showed in Scheme 2.12.[104, 143] The control step is the forming of cyclic lactone-enzyme complex, and thus gave an acyl-enzyme intermediate, which is also named as "enzyme-activated monomer" (EM). The following initiation procedure involves the nucleophilic attack of water, which is contained partly in the enzyme, onto the acyl carbon of the intermediate to produce a ω–hydroxycarboxylic acid (n=1). Then the lipase will be released from the acyl-enzyme intermediate for another ring-opening of monomer. In the propagation stage, the first linear molecule from the opened cyclic monomer will nucleophilically attack the acyl-enzyme intermediate to produce a one-unit-more elongated polymer chain.

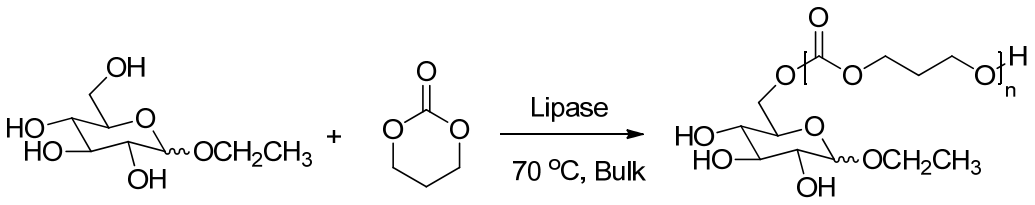


Scheme 2.12 Mechanism of Lipase-Catalyzed Polymerization of Cyclic Lactones

Lipase-catalyzed ring-opening polymerization of cyclic lactones was a versatile method to synthesize biodegradable polyesters for biomedical applications. Polyesters with well defined structure, molecular weight and corresponding physical and mechanical properties were obtained by choosing specific monomers. Lipases applied for polyester preparations showed high catalytic activity and stability in organic meida. However, the obtained polyesters from lipase-catalyzed ring-opening polymerization have a relatively low molecular weight, this may due to the relatively lower activation energy provided by enzymes or the reversible hydrolysis reactions at a relatively high monomer conversion. As an attractive and "green" catalyst for polymer synthesis, lipase catalysts have been further applied for ring-opening polymerization of cyclic carbonates.

Lipase-Catalyzed Ring-Opening Polymerization of Cyclic Carbonates:

With the similar structures of cyclic lactones, cyclic carbonates have also been targeting monomers for lipase-catalzyed ring-opening polymerization.[104, 143] Timethylene carbonate (TMC) was the most prominent monomer has been studied for lipase-catalyzed ring-opening polymerization, and the corresponding poly(trimethylene carbonate) (PTMC) is an important biodegradable and biocompatible materials.[152-154] Various lipases including lipase PS, PC, PF, CA, CC, and PPL have been used for the polymerization of TMC. Matsumura *et al.* examined PPL-catalyzed polymerization of TMC at a high temperature of 100 ^{o}C. With a lipase amount of 0.25-10 wt% involved in the reaction, Poly(trimethylene carbonate) (PTMC) with high monomer conversion of 99% with M_n up to 169,000 was achieved after 24 h. The polymers produced from immobilized PPL showed higher molecular weight of 46800 by using even lower lipase amount of only 0.05 wt%.[155] Kobayashi *et al.* and Bishit *et al.* reported lipase CA catalyzed polymerization of TMC under a mild reaction temperature of 60 to 75 ^{o}C. Molecular weight of 2000 was achieved after 3-4 days polymerization.[156, 157] Sugar-terminated PTMC was synthesized by using ethylglucoside (EGP) as multifunctional initiator for Novozym 435-catalyzed ring-opening polymerization of TMC (Scheme 2.13). EGP-ended oligomer of PTMC was formed with a molecular weight of 7200 in high monomer conversion of 97%. Moreover, the high regio-selectivity of Novozym 435 was demonstrated by using the multifunctional initiator of EGP.[158]



Scheme 2.13 Novozym 435-Catalyzed Ring-Opening Polymerization of TMC

To extend the applications of enzymatic polycarbonate preparation, some PTMC-based copolymers have been studied by lipase-catalyzed ring-opening polymerization of TMC with other cyclic monomers, such as ε-caprolactone,[159] β-butyrolactone,[160] δ-valerolactone,[160] $_L$-lactide,[160] ω-pentadecalactone,[161, 162] 5-methyl-5-benzyloxycarbonyl-1,3-dioxan-2-one (MBC),[163] and 5-methyl-5-carboxyl-1,3-dioxan-2-one (MCC).[164] The ring-opening polymerizations of TMC with cyclic lactone *co*-monomers gave poly(ester-carbonate)s containing polycarbonate and polyester units in polymer chains, which provided tunable degradation rate for being used as drug delivery devices. The one-pot bulk polymerization of TMC and MCC gave random copolymer of PTMC/PMCC with functional –COOH group on the side chain, the active side chain may be easily modified or polymerized for specific applications.

As reviewed in previous sections, lipase-catalyzed ring-opening polymerization of cyclic lactones and cyclic carbonates is an efficient method for polymer synthesis. The *non*-toxic lipase catalysts provide good biocompatability for the prepared polyesters or polycarbonates being potential materials for biomedical applications. Regarding to the ring-opening polymerization, there is no small molecule such as water or alcohol, which may cause side reactions or enzyme deactivation, released during the reaction. Thus, being an attractive method for polymer synthesis. However, due to the limitation of available cyclic monomers, other synthetic method for polymer synthesis will also be necessary to provide much wider spectrum of properties.

2.2.2 Lipase-catalyzed Polycondensation

Lipase-catalyzed polycondensation has attracted great research intensions for the polyester synthesis. In 1983, Okumura *et al.* reported the first enzymatic oligomerization of hydroxy acid during the hydrolysis of castor oil by *Geotrichum candidum* lipase, where the liberated ricinoleic acid was oligomerized to produce estolides mainly composed of dimmers and trimers.[165] Gatfield noted that the macrocyclic pentadecanolide and γ-butyrolactone may be formed when 15-hydroxypentadecanoic acid and 4-hytdroxybutyric acid were exposed to the lipase of *Mucor miehei*, respectively.[166] Other syntheses of lactones from hydroxylesters, such as ω-hydroxy acid methyl esters[167] and γ-hydroxyester,[168] have also been reported.

Lipase-Catalyzed Polycondensation of Dicarboxylic acid and Glycol

Linear polyester oligomers were formed after exposure of diacids or diesters and diols to lipase. Margolin *et al.* demonstrated the synthesis of optically active oligoesters by PPL-catalyzed polycondensation between a racemic diester and an achiral diol, or a racemic diol and an achiral diester. In both cases, trimers and pentamers of AA-BB-AA and AA-BB-AA-BB-AA types were produced.[169] The reaction between adipic acid and hexadecanediol was carried out by lipase from *Pseudomonas sp.* at 65 ^{o}C, it gave the cyclic mono-lactone in 52% yield and di-lactone in 19% yield as major products. However, the linear polyester oligomer was detected under a lower reaction temperature of 45 ^{o}C.[170] Another linear polyester prepared from 1,13-tridecanedioic acids and 1,3-propanediol was reported. The polycondensation was performed at 30 ^{o}C for 16 h by

using lipase from *Aspergillus niger* NRRL 337 as catalyst. The dominant components of synthesized esters were pentamer and heptamer, and both end groups of the pentamer and heptamer were hydroxyl group.[171]

In contrast with the produced oligomers of polyesters, high-molecular-weight polyesters were enzymatically synthesized *via* the polycondensation of sebacic acid and 1,4-butanediol under optimized reaction conditions. Linko *et al.* found that the reaction may be more efficient if the water produced during the polymerization could be effectively removed. Vacuum was also noticed to be a possible tool, which may drive the reaction to the desired direction by removing the released water or small molecules.[172, 173] Vacuum was further confirmed as an useful method to improve the polymerization. Wu *et al.* studied the preparation of aromatic polyesters by lipase-catalyzed polycondensation. A vacuum of 2.6 KPa was exerted for 10 min when the reaction proceed to 5 h and 10 h, then the vacuum was increased to 20 to 40 Pa at 22 h until the polymerization was totally completed. A much higher molecular weight of polymer was obtained, and the reaction rate was also relatively increased.[174]

The effects of different substrates and solvents on the polymer chain formation, polydispersity and end-group structure were investigated by several research groups. Uyama *et al.* reported *Candida antarctica* lipase-induced polymerization of dicarboxylic acid (C2-C12) and glycol (C2-C12) in a solvent-free system. They found that the polymerization behavior strongly depended on the chain length of both monomers.[175] In the meantime, Mahapatro *et al.* reported that monomers with longer chain length (sebacic and adipic acid / 1,8-octanediol and 1,6-hexanediol) showed higher reactivity than those with shorter chain length (succinic and glutaric acid / 1,4-butanediol). In the same

reaction system, the effect of solvents on the polymerization was claimed to be complicated, but the diphenyl ether was found to be the preferred solvent to give a higher molecular weight of the polyesters.[176] Sahoo *et al.* studied the influence of PEG ending group and chain length on it reactivity for Novozym 435-catalzyed polyester synthesis. In the polymerization between sebacic acid and PEG diols with different molecular weight (PEG 200, 400, 600, 1000, 2000, 10000), PEG400 and 600 were found to be most active monomers. A higher chain length of PEG resulted in decreased average degree of polymerization (DP_{avg}). The reaction between PEG200 diacids ($HOOC\text{-}(CH2)_x\text{-}O\text{-}(CH2CH2O)_n\text{-}(CH2)_x\text{-}COOH$) and 1,8-octanediol showed that the increase of the α,ω-carboxyalkyl methylene spacer length (x) from 1 to 5 caused the increased DP_{avg} of polymerization from 3.9 to 25.4, and the further increase of x did not bring about additional increase of DP_{avg}.[177]

Polycondensation between dicarboxyl acid and glycol is generally realized as a dehydration reaction in *non*-aqueous media, and water disfavors dehydration to precede the polymerization in an aqueous medium. However, the lipase catalysis still can provide efficient activity for the polymerization of a dicarboxyl acid and glycol in water. For instance, lipase PC and PPL were applied for the polymerization of sebacic acid and 1,8-octanediol in aqueous media, and a polyester with molecular weight of 1600 was obtained.[178] Recently, ionic liquids were also applied for polyesters synthesis as green solvents.[179] Nara et al. reported lipase PS-C (lipase *Pseudomonas cepacia* supported on Celite) catalyzed polycondensation of diethyl octane-1,8-dicarboxylate and 1,4-butanediol in 1-butyl-3-methylimidazolium ($[bmim]PF_6$) at 60 ^oC or room temperature, and polymers with molecular weight of 2000 were successfully produced.[180]

The stoichiometry played an important role in the polycondensation, it may significantly affect the polymer structure and the polymerization kinetics. The stioichiometry effect on the lipase CA-catalyzed polycondensation of adipic acid and 1,6-hexadediol was examined by Nakaoki *et al.* In case of the stoichiometric substrates, ^{1}H NMR analysis demonstrated that the OH-terminated product was mainly produced at the early stage of polymerization. As the reaction proceeded, the carboxyl-terminated polymer became the major product. Even in the case of an excess of dicarboxylic acid monomer used, the OH-terminated polymer was the major product at the early reaction stage.[181]

Lipase-Catalyzed Polycondensation of Dicarboxylic acid ester and Glycol

Dicarboxylic acid alkyl esters were another possible substrates for lipase-catalyzed polycondensation with glycols.[182-187] Alkyl esters generally showed relatively low reactivity, thus it would be difficult to produce polyesters with high molecular weight. To drive the thermodynamic equilibrium of the polymerization, adsorption of the released alcohol by molecular sieves,[184] removing alcohol by nitrogen bubbling[184] or under vacuum[172, 188] were investigated. High molecular weight of 20,000 was obtained by the lipase-catalyzed polycondensation of sebacic acid and 1,4-butanediol in diphenyl ether or veratrole under reduced pressure. The vacuum condition effectively improved the activity of dicarboxylic acid alkyl esters and produced polymers with higher degree of polymerization, but the production cost will also increase significantly.

Lipase-Catalyzed Polycondensation of Activated-Dicarboxylic acid ester and Glycol

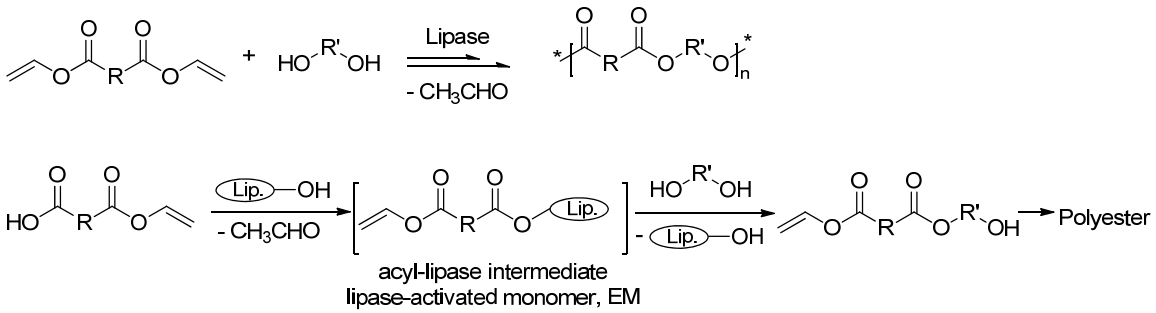
To improve the kinetics of the polycondensation reaction, the moderate molecular weight polyesters required efficient methods to shift the thermodynamic equilibrium towards the direction of polymer formation. Activated dicarboxylic acid esters have earned great intention in lipase-catalyzed polycondensation due to their relatively active ending groups. Margolin *et al.* investigated the polycondensation of bis(2-chloroethyl)(±)-2,5-bromoadipate with 1,6-hexanediol in toluene to achieve optically active polyesters from racemic diesters and achiral diols.[169] Wallace and Morrow noted halogenated monomers, such as *bis*(2,2,2-trichloroethyl)-*trans*-3-hexanedioate,[183, 189] *bis*(2,2,2-trifluoroethyl)-glutarate,[190] *bis*(2,2,2-trichloroethyl) adipate,[191] and bis(2,2,2-trifluoroethyl) sebacate,[192] activated the acyl donor and thus improved the polymerization kinetics. As an example, PPL-catalyzed enantio-selective copolymerization of *bis*(2,2,2-trichloroethyl)-(±)-3,4-epoxyadipate and 1,4-butanediol demonstrated high reactivity of the functionalized terminal groups, the M_n of the polymer was calculated as 5,300 by end group analysis from ^{1}H NMR.[189] A much higher molecular weight of M_w=46,400 was obtained by lipase MM (lipase from *M. miehei*)-catalyzed polycondensation of *bis*(2,2,2-trifluoroethyle) sebacate with 1,4-butanediol in diphenyl ether at 37 ^oC under vacuum condition. The elimination of released alcohol during the reaction was found to be an important factor to achieve a high molecular weight of polymer.[192] Halogenated dicarboxylic acid ester showed better reactivity than *un*-activated dicarboxylic acid esters in lipase-catalyzed polycondensation. This improvement may due to the halogen atoms playing as stronger electron-donors. An increased degree of polymerization with higher

molecular weight of polymer was achieved from the polycondensation of halogenated dicarboxylic acid esters and diols, which has been of an efficient method for polyesters synthesis.

An irreversible procedure for lipase-catalyzed acylation using enol esters as acylating reagents was developed for polyester synthesis by Uyama and Kobyashi.[193] They reported the first example of lipase-catalyzed polyester synthesis from divinyl adipate and glycol. The use of divinyl ester provided an irreversible polymerization because the released vinyl alcohol can be rapidly rearranged to acetaldehyde, which may be easily eliminated from the reaction system. This help shift the thermodynamic equilibrium to the polymer formation direction. Chaudhary *et al.* explored Novozym 435-catalyzed bulk polymerization of divinyl adipate and 1,4-butanediol at 50 ^{o}C for 4 h. A high molecular weigh of M_w=23,236 was achieved with a reaction extent of 98.3%.[194] Divinyl esters of dicarboxylic acids, including isophathalic acid, terephthalic acid, *p*-phenylene diacetic acid, sebacic acid, have also been studied with glycols for lipase-catalyzed polycondensation. Various lipases, such as lipases from *Candida antarctica*, *Candida cylinderacea*, *Mucor meihei*, *Pseudomonas cepacia*, *Peudomonas fluorecens* and *Porcine pancreas*, in heptane at 60 ^{o}C for 48 h. Among these lipases, lipase *Candida antarctica* showed the highest activity and gave the polymer with the highest molecular weight of 5500 in 74% yield.[195]

The mechanism of lipase-catalyzed polycondensation of divinyl ester and glycol was proposed as shown in Scheme 2.14. The serine residue nucleophilically attacks the acyl-carbon of the divinyl ester to form an acyl-lipase intermediate. In the meantime, acetaldehyde will be eliminated immediately. Then the acyl-lipase intermediate reacts

with glycol to produce the first bonding unit containing both monomers. In the propagation stage, the nucleophilic attack of the terminal hydroxy group takes place on the acyl-lipase intermediate formed from the vinyl ester group of the monomer, and then the propagation steps proceed similarly.

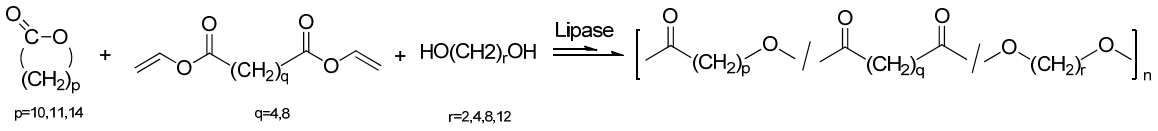


Scheme 2.14 Mechanism of Lipase Catalyzed Polycondensation of Divinyl ester and Glycols

During the lipase-catalyzed polycondensation of divinyl esters and glycols, there was a competition between the transesterification and hydrolysis of the vinyl end group. This hydrolysis reaction may significantly limit the growth of polymer chain, thus resulted in lower molecular weight. The polymerization was controlled by the relative magnitudes of the rate of transesterification and hydrolysis.[194] Chaudhary *et al.* established a mathematical model based on experimental data to simulate and predicted the polymerization kinetics. They found that the molecular weight and terminal groups of polyesters were strongly influenced by the selective nature of different biocatalysts.[196] The monomer concentration in the reaction system may also have significant effect on the polymerization rate. The reaction rate constant was relatively high at the early stage of polymerization, while it was dramatically reduced as the polymerization proceeded.

Both lipase-catalyzed ring-opening polymerization and polycondensation have been widely applied for polyester synthesis.[104, 143, 197-199] Based on the mechanism

analysis, lipase-catalyzed polycondensation is considered to proceed *via* the similar reaction intermediate (acyl-enzyme intermediate) with that of lipase-catalyzed ring-opening polymerization. Thus, the lipase-catalyzed ring-opening polymerization and lipase-catalyzed polycondensation were combined to produce polyester copolymers. Kobayashi and his coworker reported the first example for polyester synthesis through the combination of ring-opening and condensation polymerizations. Lipase PC-catalyzed co-polymerization of PDL, divinyl sebacate, and 1,4-butanediol in *i*-propyl ether was performed at 60 ^{o}C for 72 h. In this copolymerization, two different types of polymerization, ring-opening polymerization and polycondensation, simultaneously occurred *via* the same reaction mechanism. The copolymer was obtained with a molecular weight of 6,500 in 80% yield.[135] Similar work was carried out by Namekawa *et al.* By using different lipases, they studied the preparation of polyesters from lactones, dicarboxylic acid divinyl esters and glycols through combination of ring-opening polymerization and polycondensation (Scheme 2.15).[200] Lipase CA and lipase PC showed relatively higher reactivity for the present polymerization. The polymer yield and molecular weight were observed to have a clear relationship on the monomer combination and feed ratio.



Scheme 2.15 Polyester Synthesis by Combination of Lipase-Catlayzed Ring-Opening Polymerization and Polycondensationl

2.2.3 Enzymatic Modification of PHB

As mentioned previous sections, enzymatic ring-opening polymerization of cyclic lactones or carbonates have been widely studied and applied for the polyesters or polycarbonates synthesis. Some PHB-based copolymers were synthesized by the ring-opening polymerization of *β*-butyrolactone with different monomers. Jedlinski *et al.* studied PPL-catalyzed copolymerization of *β*-butyrolactone with 12-hydroxydodecanoic acid at 45 ^{o}C for 72 h in toluene, chloroform and disiopropyl ether, respectively. The prepared copolymers with M_n of about 2000 were obtained in 51-70% yield.[201] Kikuchi *et al.* investigated the lipase CA-catalyzed copolymerization of *β*-butyrolactone with different *non*-substituted lactones, such as *ε*-caprolactone, 11-undecanolide, 12-dodecanolide, and 15-pentadecanolide, at 60 ^{o}C. From these reactions, PHB-based random copolymers were synthesized with molecular weight ranged from 2000 to 9300 in 20-48% yield.[202] However, the examples for the PHB-based copolymers synthesis are still limited. Furthermore, the random copolymer can not show the competitive biocompatible and biodegradable properties of natural-produced PHB. There was no example for the enzymatic synthesis of PHB-based block copolymers with microbial PHB or functionalized microbial PHB as starting material.

2.3 Candidates for Microbial PHB-based Block Copolymers

Polymeric materials have been studied for biomedical applications and some of them have been practically applied as biomaterials. Different structures of polymer chains with different chain lengths provided wide spectrum of physical and mechanical properties to favor the specific requirements of clinical applications. With the rapid

development in direct tissue replacement and tissue engineering, the synthesis of degradable biomaterials became a relatively new area of research. An overview of some degradable polymers and their current applications are summarized in Table 1.3. Degradable polyesters were the most prominent biomaterials, and they were adopted in surgery around 30 years ago as materials for sutures and bond fixation devices.[203] Poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and poly(ε-caprolactone) (PCL) are in common clinical use and are characterized by degradation time ranging from days to years depending on the formulation and initial molecular weight.

Lactic acid is a chiral molecule, existing in $_{L}$ and $_{D}$ isomers (the $_{L}$ isomer is the biological metabolite), and thus poly(lactic acid) refers to the family of: poly($_{L}$-lactic acid) (PLLA), poly($_{D}$-lactic acid) (PDLA) and poly($_{D,L}$-lactic acid) PDLLA. PLLA and poly(ε-caprolactone) are widely used clinically, and can be degraded by hydrolytic attack of the ester bond. The physical and mechanical properties, as well as the degradation behavior are affected by the complicated effects of the crystallinity, the molecular weight, the glass transition temperature and the monomer hydrophobicity.[204-208] PGA is the simplest linear aliphatic polyester. It is highly crystalline and has a T_m of 160 ^oC and a T_g of 35 ^oC, which is quite close to body temperature.[209] To adapt the properties of PGA, copolymer containing PLA were intensively studied. PLA is a semi-crystalline polymer with a T_m of 60 ^oC and a T_g of 55 ^oC.[207] The hydrophobicity of PLA prevents the water uptake of thin film to about 2% and reduces the rate of backbone hydrolysis compared with those properties of PGA. Thus the copolymer of PLGA may have a wider range of physico-mechanical properties and degradation behavior. However, there is no linear relationship between the ratio of PLGA and the physico-mechanical properties. The high crystallinity

of PGA was reported to be rapidly decreased in PLGA copolymer, which led to an increase of the hydration and hydrolysis rate of the copolymer. Thus typically 50:50 PLGA copolymer degrade more rapidly than either PLA or PGA.[209]

Table 2.1 Degradable Polymers and Their Current Applications.[215]

Degradable Polymers	Current Applications
Synthetic Degaradable Polymers	
Poly(glycolic acid), poly(lactic acid) and copolymers	Barrier membranes, drug delivery, guided tissue regeneration, orthopedic applications, stents, staples, sutures, tissue engineering
PHB and Polycaprolactone Copolymer	Long-term drug delivery, orthopedic application, stents, staples
PHB and Polydioxanone Copolymer	Fracture fixation in non-load-bearing bones, sutures, wound clip
Polyanhydrides	Drug delivery
Polycyanoacrylates	Adhesives, drug delivery
Poly(amino acids) and “pseudo”-Poly(amino acid)	Drug delivery, tissue engineering, orthopedic applications
Poly(ortho ester)	Drug delivery, stents
Polyphosphazenes	Blood contacting devices, drug delivery, skeletal reconstruction
Poly(propylene fumarate)	Orthopedic application
Natural Polymers	
PHB, PHV and PHBV	Long-term drug delivery, orthopedic application, stents, sutures
Collagen	Artificial skin, coatings to improve cellular adhesion, drug delivery, guided tissue regeneration in dental applications, orthopedic applications, soft tissue augmentation, tissue engineering, scaffold for reconstruction of blood vessels, wound closure
Fibrinogen and fibrin	Tissue sealant
Gelatin	Capsule coating for oral drug delivery, hemorrhage arrester
Cellulose	Adhension barrier, hemostat
Various polysaccharides such as chitosan, alginate	Drug delivery, encapsulation of cells, sutures, wound dressings
Starch and amylose	Drug delivery

The major disadvantages of PGA, PLA and PLGA include the degradation products reduce the local pH value, and the limited mechanical properties for hard tissue regeneration.[210, 211]

PCL is also a semi-crystalline and highly thermal-stable polyester with a low T_m of around 60 ^oC and a T_g of -60 ^oC.[212] PCL is always in a rubbery state at room temperature, which contributes to the high permeability of PCL for many therapeutic drugs. [210, 213, 214] Moreover, PCL has a relatively slower degradation rate than PLA, and it is currently regarded as a *non*-toxic and tissue compatible polyester, thus can be used in drug delivery devices that remain active for over a year.

Poly[(*R*)-3-hydroxyoctanoate] (PHO) is a representative of medium-chain-length-PHA (*mcl*-PHA) with a C5 side chain on its backbone. It is a soft-sticky polyester with a low T_m of 61 ^oC and a T_g of -30 ^oC. The weak crystallinity make it can not be directly processed. However, PHO may be a desired material to be used as soft segments in a block copolymer.

Aliphatic polycarbonates have also earned great interest as environmentally benign materials over the past three decades. Poly(trimethylene carbonate) (PTMC) is one of the polycarbonates that have been studied for their potential use in biomedical applications. PTMC is a rubbery and elastomeric material with no T_m and a T_g of about -20 ^oC, low Young's modulus of 3-7 MPa and elongation at break up to 1000%.[216-218] Many efforts have been investigated to obtain copolymers of PTMC to achieve better mechanical properties and different degradation rate.

Although there have been several families of polymers studied for biomedical applications, and some of them have been commercially available, the available

biopolymers are still too limited to cover the diverse properties requirements of different biomedical applications. The design and synthesis of novel polymeric biomaterials is currently of important research interests and challenge.

CHAPTER 3

ENZYMATIC PREPARATION OF NOVEL THERMOPLASTIC *di*-BLOCK CO-POLYESTERS CONTAINING POLY[(*R*)-3-HYDROXYBUTYRATE] AND POLY(ε-CAPROLACTONE) BLOCKS *via* RING-OPENING POLYMERIZATION

3.1 Introduction

As mentioned in previous chapter, microbial poly[(*R*)-3-hydroxybutyrate] (PHB) is the most prominent polyester in the PHA family, and can be easily produced in large quantity. PHB may be useful for drug delivery or tissue engineering, but its application as thermoplastic material is rather limited partially due to the high melting temperature (T_m) of 175-177°C and high glass transition temperature (T_g) of 4°C.[1, 219] Many methods have been used to prepare PHB-based copolymers to improve the thermoplastic properties. Bacterial syntheses by feeding with different substrates allowed for the preparation of random co-polyesters containing PHB and other PHA members such as poly[(*R*)-3-hydroxyvalerate] (PHV),[14] poly[(*R*)-3-hydroxypropionate] (PHP),[66] poly[(*R*)-3-hydroxyhexanoate],[67] or poly[(*R*)-3-hydroxydecanoate].[68] Chemical modification of PHB led to the preparation of block-co-polyesterurethane[82-84, 87] and block-co-polyesters[85] containing the hard PHB block and other soft blocks such as poly(ε-caprolactone) (PCL),[83] poly[(*R*)-3-hydroxyoctanoate],[84, 85] or poly(ethylene glycol)[87] blocks. A random co-polyester poly(HB-*co*-CL)[220] was also prepared by chemical modification of PHB. Some of these co-polymers demonstrated good thermoplastic properties. Nevertheless, microbial syntheses generally give random co-polyesters with relatively high production costs; and chemical modifications involve the use of toxic chemicals or catalysts.

We are interested in the enzymatic modification of microbial polyesters such as PHB to prepare block-co-polymers with good thermoplastic properties for biomedical applications. Enzyme catalysis is highly chemo-, regio-, and stereo-selective, thus being a

useful tool for the preparation of polymers with novel structures. Enzyme catalysis is *non*-toxic, which is very attractive for the preparation of polymeric biomaterials. Many enzymes have been used for the preparation of natural or unnatural materials,[95] and lipase-catalyzed ring-opening polymerizations (ROP) represent prominent examples.[104, 112, 143] Enzymes such as *Porcine pancreatic* lipase,[112, 139, 151] *Pseudomonas sp.* lipase,[119] and *Candida antarctica* lipase B (CALB)[124, 136, 140, 148] have been successfully used for the ROP of lactones with alcohols, diols, and polyols as initiators to prepare polyesters. Nevertheless, enzymatic ROP has not yet been applied for the modification of microbial polyesters.

Previously Suter,[82] Andrade,[84, 85] and Li Xu[87] successfully utilized low-molecular-weight telechelic hydroxylated PHB (PHB-diol) as hard segment for chemical preparation of thermoplastic block-co-polymers. PHB-diol containing a primary and a secondary OH end group might also be a suitable initiator for enzymatic ROP. While both OH end groups were reacted in chemical polymerization, the primary and the secondary OH end groups may show different reactivity in a lipase-catalyzed ROP. Thus, it could be possible that only the primary OH of the PHB-diol initiates the ROP giving a *di*-block copolymer with the unreacted secondary OH group as an end group. On the other hand, PCL is a biodegradable and biocompatible soft material with a T_m of 60°C and T_g of -60°C.[212] Although random co-polyester poly(HB-*co*-CL) prepared from PHB and PCL *via* enzyme-catalyzed transesterification[221, 222] did not show improved elastic properties, incorporation of PCL block into PHB-based block co-polymers should significantly improve the elastic properties, which has been demonstrated in the chemically prepared block co-polyesterurethanes. Recently, we have explored, for the

first time, the lipase-catalyzed ROP of ε-caprolactone with PHB-diol to prepare block co-polyester poly(HB-*co*-CL). Here we studied the new and selective enzymatic ROP, the preparation of the corresponding block copolymers, the structural analysis of the novel *di*-block co-polyesters, and the characterization of physical properties of the polymers.

3.2 Experimental Section

Materials. Novozym 435 (immobilized *Candida antarctica* lipase B, 10000 PLU/g) was purchased from Novozymes. Telechelic hydroxylated poly-[(*R*)-3-hydroxybutrate], PHB-diol(M) (M_n of 3000, GPC, single peak), was a gift from Dr. P. Neuenschwander at ETH Zurich. Microbial PHB (>99%), dibutyltin dilaurate (95%), diglyme (99.5%), ε-caprolactone (99%), 1,4-dioxane (99.8%), and toluene (99.8%) were purchased from Aldrich. Ethylene glycol (99%), chloroform (GC, >99%), and *n*-hexane (HPLC, 99%) were obtained from Merck. Novozym 435 and PHB-diol were dried under vacuum at 50°C for 12 hours before use. ε-Caprolactone was freshly distillated over CaH_2 (83°C, 1.7 mmHg). 1,4-Dioxane and toluene were dried by refluxing over Na / benzophenone under argon.

Telechelic hydroxylated poly-[(*R*)-3-hydroxybutrate], PHB-diol(P): PHB-diol (P) was prepared using the known procedure for the preparation of PHB-diol (M) with large excess of ethylene glycol to PHB. Transesterfication of PHB (8.0 g) and ethylene glycol (40 mL) in the presence of dibutyltin dilaurate (118 mg) in diglyme (32 mL) was performed at 135 ^oC for 2 h. The mixture was then poured into cold water, and the

precipitate was collected and washed by water (50 ml x 3) to remove the excess ethylene glycol. The crude product was dissolved in chloroform followed by precipitation at 4 ^{o}C by the addition of *n*-hexane. The product was separated by filtration and dried at 50^{o}C under vacuum for 12 h to give 6.2 g of PHB-diol (P) in 76% yield. The molecular weight (M_n) was determined by GPC as 3700 g/mol with M_w/M_n of 1.38. The physical properties were determined by DSC with T_m of 149 and 134°C and T_g of -5.0°C. The chemical structure was identified by ^{1}H-NMR as a PHB-diol with a primary OH group on one end and a secondary OH group on the other end.

General procedure of enzymatic ring-opening polymerization of *ε*-caprolactone with PHB-diol: Novozym 435 (20-160 mg immobilized enzyme) and PHB-diol(M) (M_n of 3000, GPC; 88-212 mg) or PHB-diol(P) (M_n of 3700, GPC; 33-186 mg) were added to a dry schlenk tube containing a magnetic stirring bar and activated 4Å molecular sieves, and then dried at 50°C under vacuum for 12 h. Under argon atmosphere, the freshly distillated *ε*-caprolactone (400-600 mg) and freshly dried 1,4-dioxane or toluene (1.2 g-3.6 g) was added into the schlenk tube using a dry syringe. The mixtures were stirred under argon atmosphere at room temperature, 50°C, or 70°C and samples (50 μL) were taken at regular time intervals and analyzed by GPC. The reaction was stopped at 8-48 h, 10 mL chloroform was added, and the enzyme was removed by filtration. The solvent in the filtrate was removed under reduced pressure, and the product was dissolved in chloroform and then precipitated by adding *n*-hexane or methanol. The results are summarized in Table 3.1 and 3.3.

Preparation of poly[(*R*)-3-hydroxylbutyrate(56wt%)-*co*-ε-caprolactone(44wt%)]: According to the procedure described above, reaction of PHB-diol(M) (M_n of 3000, GPC; 212 mg) and ε-caprolactone (409 mg) with Novozym 435 (40 mg immobilized enzyme) as catalyst was carried out in 1,4-dioxane (1.6 g) at 70°C, and samples (50 μL) were taken at regular time intervals and analyzed by GPC. After 48 h polymerization, the reaction was terminated by the addition of 10 mL chloroform followed by the removal of Novozym 435 through filtration. 1,4-dioxane and chloroform were removed by evaporation under reduced pressure. The raw product was dissolved in 2 mL chloroform, treated with 18 mL methanol, and then precipitated at 4°C for 12 h. After removal of the solvent by filtration, the precipitate was dried at first by evaporation under vacuum and then in a vacuum oven at 50°C for 24 h. This gave 410 mg (66% yield) of the polymer. The molecular weight M_n was determined by GPC as 5400 (M_w/M_n of 1.63), and the structure was analyzed by NMR and IR as *di*-block co-polyester poly(HB-*co*-CL) with two different OH end groups. The ratio of PHB and PCL block was established as 56/44 (wt/wt) based on M_n (NMR) of PHB-diol(M) and the polymer. The physical properties were determined by DSC and WAXD with T_m of 145°C, 123°C, and 53°C, T_g of -57°C, and crystallinality of 19%.

Preparation of poly[(*R*)-3-hydroxylbutyrate(28wt%)-*co*-ε-caprolactone(72wt%)]: According to the procedure described above, reaction of PHB-diol(P) (M_n of 3700, 185 mg) and ε-caprolactone (632 mg) with Novozym 435 (76 mg immobilized enzyme) as catalyst was carried out in toluene (1.8 g) at 70°C, and samples (50 μL) were taken at regular time intervals and analyzed by GPC. After 16 h polymerization, the reaction was

terminated by the addition of 10 mL chloroform followed by the removal of Novozym 435 through filtration. Toluene and chloroform were removed under reduced pressure with a rotary evaporator. The raw product was dissolved in 2 mL chloroform, treated with 18 mL methanol, and then precipitated at 4°C for 12 h. After removal of the solvent, the precipitates were dried at first by evaporation under vacuum and then in a vacuum oven at 50°C for 24 h. This gave 702 mg (86% yield) of the polymer. The molecular weight was determined by GPC as 7900 (M_w/M_n of 1.90), the structure was analyzed by NMR and IR as *di*-block co-polyester poly(HB-*co*-CL) with two different OH end groups. The ratio of PHB and PCL block was established as 28/72 (wt/wt) based on M_n (NMR) of PHB-diol(P) and the polymer. The physical properties were determined by DSC with T_m of 149°C and 54°C, and T_g of -61°C.

Gel permeation chromatography (GPC). Molecular weight analysis (M_n and polydispersity index M_w/M_n) was performed by using a Waters instrument, with Waters 510 pump, Waters 410 refractive index detector, and Waters HR4E, HR5E and HR6 columns placed in series. THF was used as the eluent at a flow rate of 1.0 mL/min at 30°C. Sample concentration was about 0.1% (w/v) and the injection volume was 100 μL. Polystyrene standards with molecular weights of 1310, 2970, 13900, 30200, 197000 and 696000 g/mol were used to generate a calibration curve.

Nuclear magnetic resonance (NMR). ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded with a Bruker AMX500 NMR instrument in $DMSO_{d6}$ at 333K. Chemical shifts were referred to TMS at 0 ppm.

Differential scanning calorimetry (DSC). The thermal properties of polymers were measured on a Mettler Toledo DSC 822 system. Nitrogen was used as purge gas with a flow rate of 30 ml/min. Samples of 10 mg were prepared in aluminum foils, where the aluminum weights of the sample and reference were closely matched. The samples were heated from room temperature to 180 °C with a heating rate of 20 °C/min, cooled down to -100 °C with a cooling rate of -20 °C/min, and heated again from -100 °C to 180 °C at a heating rate of 20°C/min. T_m and T_g of the samples were obtained from the second heating curves.

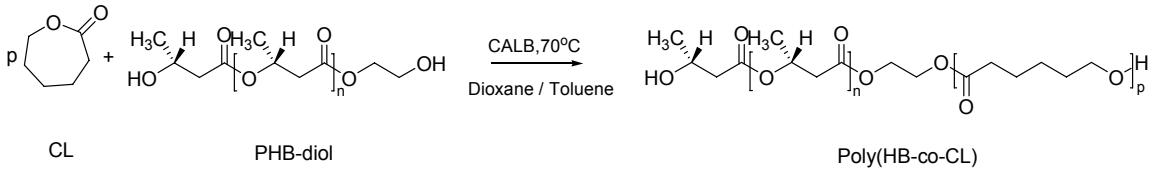
Wide angle X-ray diffraction (WAXD). The crystallinity analysis was preformed with a SHIMADZU 6000 X-ray diffractometer with Cu Kα radiation at 40 kV and 30 mA in a 2θ range of 5-40 ^{o} at scanning speed of 1.2 ^{o}/min.

Fourier transform infrared spectrophotometer (FTIR). IR spectra of the polymers were analyzed with a SHIMADZU FTIR-8400 system using potassium bromide (KBr) pressing.

3.3 Results and Discussion

Enzymatic ring-opening polymerization of ε-caprolactone with PHB-diol(M)

Reaction conditions. The enzymatic ring-opening polymerization of ε-caprolactone (CL) was initially investigated with PHB-diol (M) as an initiator (Scheme 3.1). PHB-diol(M) is a telechelic hydroxylated PHB with a M_n of 3000 g/mol (GPC) prepared by transesterification of PHB and ethylene glycol.[82] Novozym 435 [immobilized *Candida antarctica* lipase B (CALB)] was chosen as catalyst, as it is well known with high catalytic activity and good solvent resistance for the ROP of lactones.[104, 143] The polymerization temperatures were examined from room temperature to 70°C, since Novozym 435 has the highest catalytic activity at around 70°C. 1,4-Dioxane and toluene were known solvents for enzymatic ROP, and they have a boiling point of 103°C and 110°C, respectively, and good solubility for PHB-diol, thus being selected as solvents in our experiments. To avoid water-initiated ring-opening polymerization, solvent was dried before use and the reaction was carried out at anhydrous conditions under Argon atmosphere.



Scheme 2.1 Enzyme-catalyzed ring-opening polymerization of ε-caprolactone with PHB-diol.

In order to study the effects of different reaction conditions on the polymerizations, a set of experiments were designed with different weight ratios of CL/solvent, CL/enzyme, CL/PHB-diol and different reaction temperature (Table 3.1). The reactions were performed in 1,4-dioxane and followed by taking samples at different time points to determine the molecular weight by GPC. As an example, the course of polymerization in experiment 11 in Table 3.1 was shown in Figure 3.1: the number average molecular weight (M_n) of the samples taken at 4 h, 8 h, 24 h and 48 h reached 3000, 4700, 4900, 6700, and 8400 g/mol, respectively. The molecular weight and yield of final polymers under different reaction conditions are summarized in Table 3.1.

In experiments 1 to 3 (Table 3.1), three different ratios of CL/enzyme (E) were studied at 70°C with fixed ratios of CL/PHB-diol and CL/Solvent, and polymerizations are shown in Figure 3.2(i). The highest molecular weight of the resulting product was obtained at CL/E in a weight ratio of 5:1. Within the range of 20-5:1, the molecular weight of the resulting polymers increased with catalyst amount. To reduce the amount of enzyme involved while achieving a high molecular weight of the polymer, the ratio of CL/E of 8/1 was used in the rest of experiments. In experiment 4-6 (Table 2.1), CL/solvent was examined in the weight ratio of 1:3, 1:6 and 1:9 at 70°C with fixed ratios of CL/PHB-diol and CL/E. As shown in Figure 3.2(ii), the use of less solvent resulted in higher molecular weight of the polymers, probably due to higher concentration of reacting species. On the other hand, the ratio of CL/solvent should not be too small, since sufficient amount of the solvent is required to dissolve PHB-diol. The effect of the

Table 3.1. Ring-opening polymerization of ε-caprolactone with PHB-diol(M) catalyzed by Novozym 435 in dioxane.

No.	CL:**E** [a]	CL:diol	CL:**Sol** [b]	Temp.	Time	M_n (GPC)[c]	M_w/M_n	Yield	CL Conv.	Code
	wt:wt	mol:mol	wt:wt	°C	h	g/mol		%	%	
1	5:1	100:1	1:4	70	48	5500	1.65	63	53	
2	10:1	100:1	1:4	70	48	4500	1.56	57	47	
3	20:1	100:1	1:4	70	48	4000	1.48	51	40	
4	8:1	100:1	1:3	70	48	6700	1.68	63	54	
5	8:1	100:1	1:6	70	48	4300	1.53	64	55	
6	8:1	100:1	1:9	70	48	3400	1.38	64	55	
7	8:1	100:1	1:3	70	48	6700	1.79	64	53	A
8	8:1	100:1	1:3	50	48	4100	1.66	65	56	
9	8:1	100:1	1:3	RT	48	3600	1.55	69	59	
10	8:1	50:1	1:3	70	32	5400	1.63	66	49	B
11	8:1	75:1	1:3	70	48	8400	1.66	58	45	C

a: **E** for immobilized enzyme, Novozym 435. b: **Sol** for solvent, Dioxane c: Polystyrene was used as standards in GPC measurement

Figure 3.1. GPC chromatograms of block poly(HB-*co*-CL) formed at different time from experiment 11 in Table 3.1.

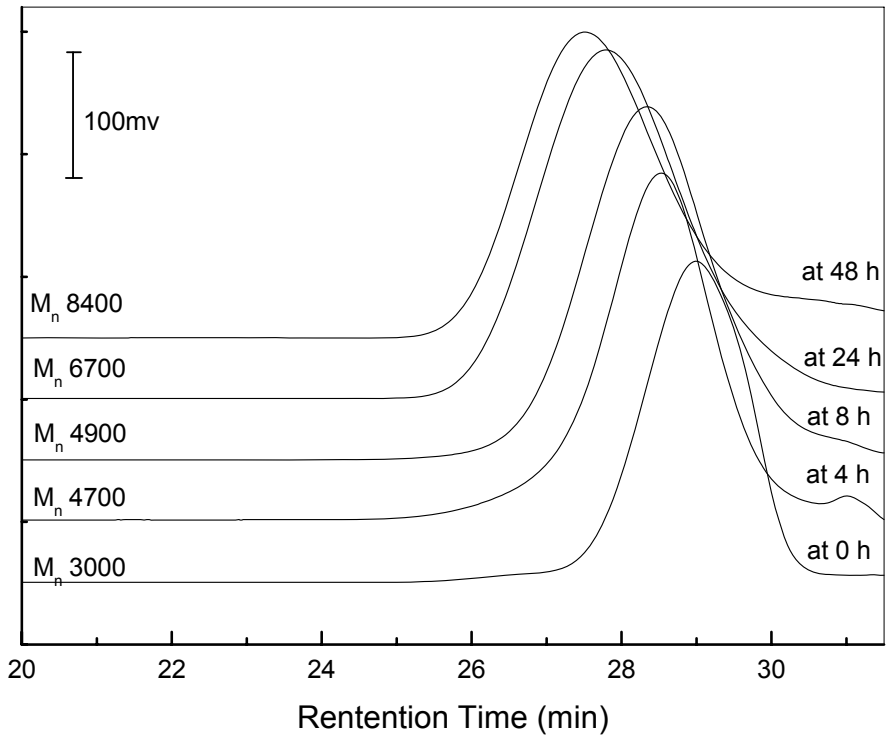
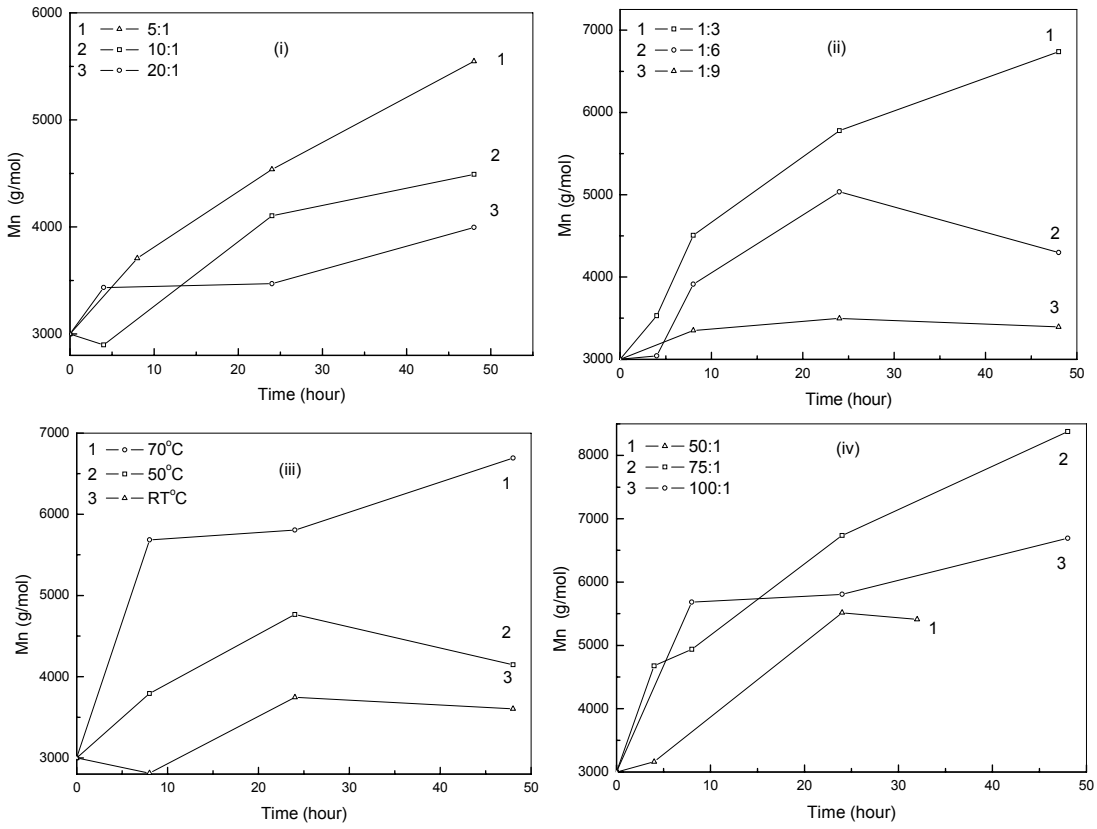


Figure 3.2. Enzyme-catalyzed ring-opening polymerization of ε-caprolactone with PHB-diol(M) under different reaction conditions: (i) different ratio of CL/Enzyme; (ii) different ratio of CL/Solvent; (iii) different reaction temperature; (iv) different ratio of CL/ PHB-diol(M).

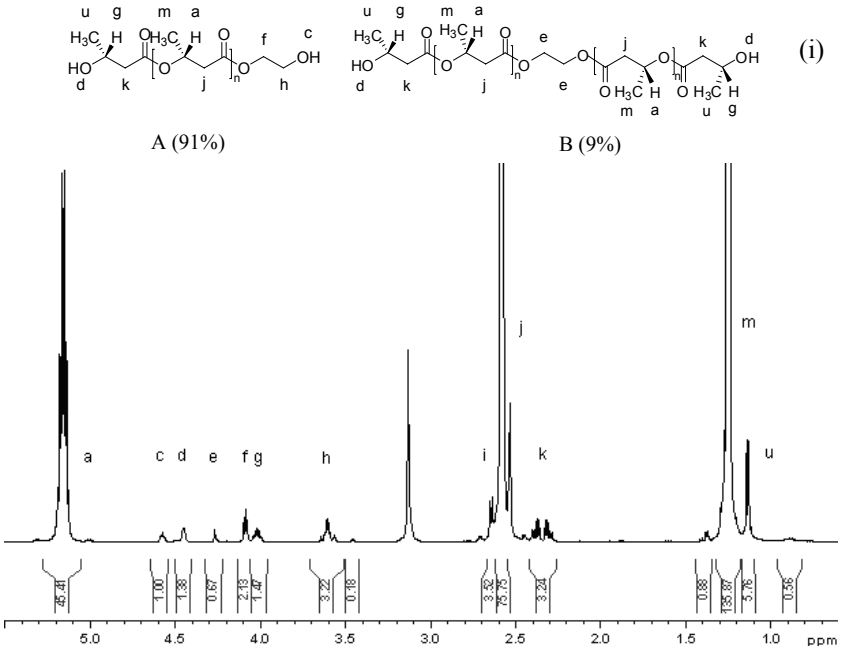


reaction temperature on the polymerization was investigated in the experiments 7-9 (Table 3.1) at rt, 50°C and 70°C, respectively. Figure 3.2(iii) clearly demonstrated that higher temperature resulted in higher molecular weight of the polymers. These results are in close correlations to the reported best temperatures of 65-70 °C for ROP with Novozym 435. Finally the effect of the molar ratio of CL/PHB-diol was investigated in experiment 7, 10, and 11 (Table 3.1) at 70°C with fixed ratios of CL/E (8:1) and CL/Solvent (1:3). As shown in Figure 3.2(iv), the highest molecular weight of the resulting polymer was obtained with a molar ratio of CL/PHB-diol in 75:1. The use of more PHB-diol could create more opportunity for the opening of CL. However, this could be in competition with the chain growth by CL, resulting in possible decrease of the molecular weight of final products. From these experiments, the best conditions examined so far for achieving highest molecular weight was 70°C, a weight ratio of CL/enzyme/solvent of 8:1:24, and a molar ratio of CL/PHB-diol of 75:1.

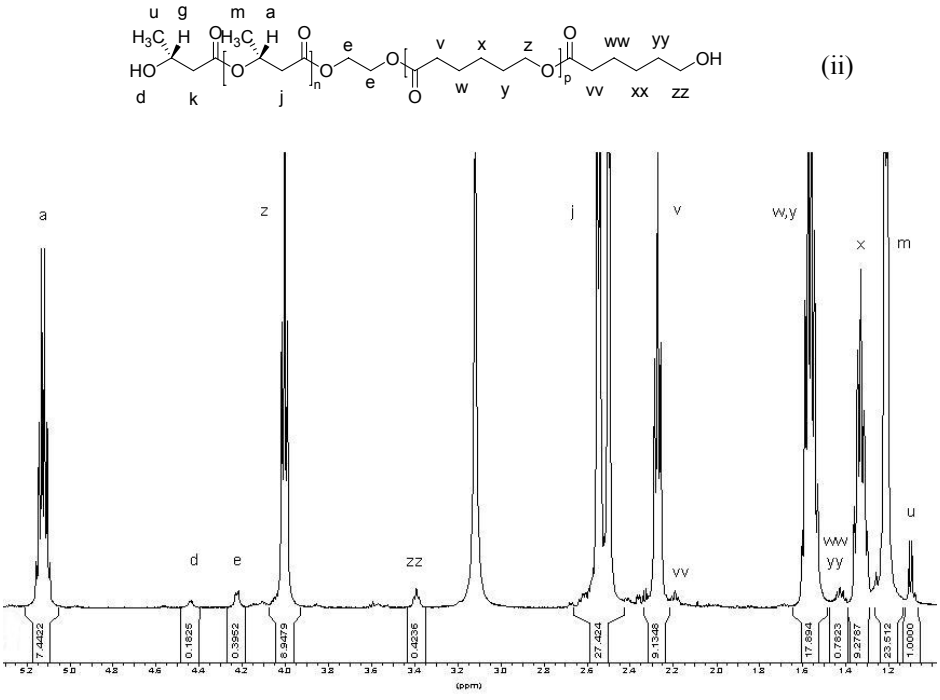
Polymer isolation. The polymerization products from all experiments in Table 3.1 were isolated by adding chloroform to the reaction mixture, removing enzyme through filtration, evaporating 1,4-dioxane and chloroform at reduced pressure, and precipitating in chloroform/*n*-hexane or chloroform/methanol (1:9). Drying the precipitates under high vacuum at 50°C for 24 h gave the corresponding block co-polyesters with M_n (GPC) of 3600-8,400g/mol in 51-69% yields and 40-59% CL conversion, respectively.

Structure analysis. The chemical structure of PHB-diol(M) was determined by ^{1}H-NMR spectrum in Figure 3.3(i). Two different structures A and B were found in PHB-diol(M).

Figure 3.3. ^{1}H-NMR spectra in $DMSO_{d6}$ at 333K: (i) PHB-diol(M); (ii) Poly[HB(56wt%)-*co*-CL(44wt%)] (sample B in Table 3.1).



(i)



(ii)

All signals were assigned to the different protons in structures A and B according to the literature.[82] Two different proton signals for primary OH and secondary OH were observed at 4.58 ppm (*c* proton) and 4.45ppm (*d* proton), respectively. The molar ratio of structures A and B was estimated as 91%:9% based on the intensities of *h* proton and *u* proton, and the number of repeat unit n was deduced as 23.6 based on the ratio of the intensities of *m* and *u* protons. The M_n of PHB-diol(M) can be thus established as 2380 g/mol which is comparable with the M_n of 3000 g/mol determined by GPC. In fact, the M_n obtained from NMR is more reliable, since the M_n determined from GPC was based on the use of polystyrene as standard.

The chemical structures of the polymers were determined by NMR and IR spectra. The ^{1}H-NMR spectrum of sample B (Table 3.1) was shown in Figure 3.3(ii). The *c* proton (primary OH, δ = 4.58 ppm) of PHB-diol(M) disappeared and the *d* proton (secondary OH, δ = 4.45ppm) was clearly observed. This indicates that the primary OH end group of PHB-diol(M) is more reactive and hence totally reacted, while the secondary OH end group is less reactive thus remaining in the polymer. The signal of *h* proton (δ = 3.56ppm) and *f* proton (δ = 4.10ppm) disappeared, which suggested again that all primary OH groups of PHB-diol(M) were reacted. The polymerization was further evidenced by the increased intensity of *e* proton. While reaction with a primary OH end group of PHB-diol(M) did not change the ratio of *m*/u protons, polymerization with a secondary OH end group would transform a *u* proton into a *m* proton, thus increasing the ratio of *m*/*u* protons. From Figure 3.3 (ii), the ratio of *m*/*u* in the polymer was calculated as 23.5 which was the same as the *m*/*u* ratio for the starting material PHB-diol(M). Thus

it is unlikely that the secondary OH end group was reacted in the ROP. As a result, the polymer is a *di*-block. The signals of PCL block were also assigned in Figure 3.3 (ii). With the ratio of *z*/*a* of 1.20:1, the number of repeating unit p in PCL block was calculated as 14.2. The molecular weight of poly(HB-*co*-CL) could thus be established as 3900 g/mol. This value is smaller than the M_n of 5400 g/mol determined by GPC. But, as it was mentioned above, the M_n obtained from NMR is more reliable.

The block copolymer structure was further evidenced by the ^{13}C-NMR spectrum in Figure 3.4. In the area of 160-180 ppm, only two signals at 168 and 172 ppm were observed and they were assigned to the carbonyl groups in PHB block and PCL block, respectively. From previous report[220] and our control experiment of lipase-catalyzed transesterification of PLC and PHB-diol, random polymer poly(HB-*co*-CL) contains at least two different types of carbonyl groups which absorbed at 169 and 171 ppm between the two signals at 168 and 172 ppm. In the ^{13}C-NMR spectrum of our polymer, no such signals can be detected. This indicated no random polymer formed during the polymerization.

In the IR spectra in Figure 3.5(i) and (iii), the primary and the secondary terminal OH group of the starting material PHB-diol(M) showed two different absorptions at 3437 cm^{-1} and 3538 cm^{-1}. There were also two absorptions at 3438 cm^{-1} and 3533 cm^{-1} for the OH groups in the block co-polymer. This further confirmed that poly(HB-*co*-CL) is a *di*-block polymer containing a primary and a secondary OH group. In the case of A-B-A

Figure 3.4. ^{13}C-NMR spectra of Poly[HB(56wt%)-*co*-CL(44wt%)] (sample B in Table 3.1) in $DMSO_{d6}$ at 333K.

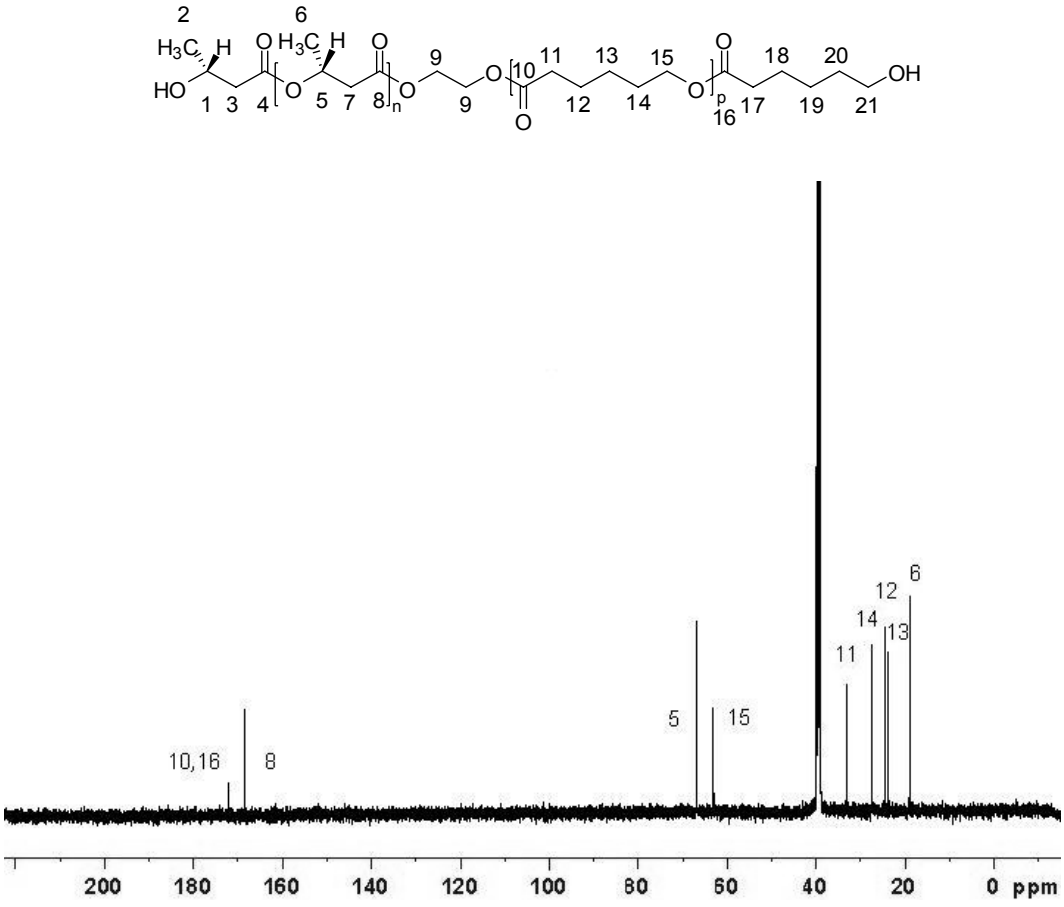
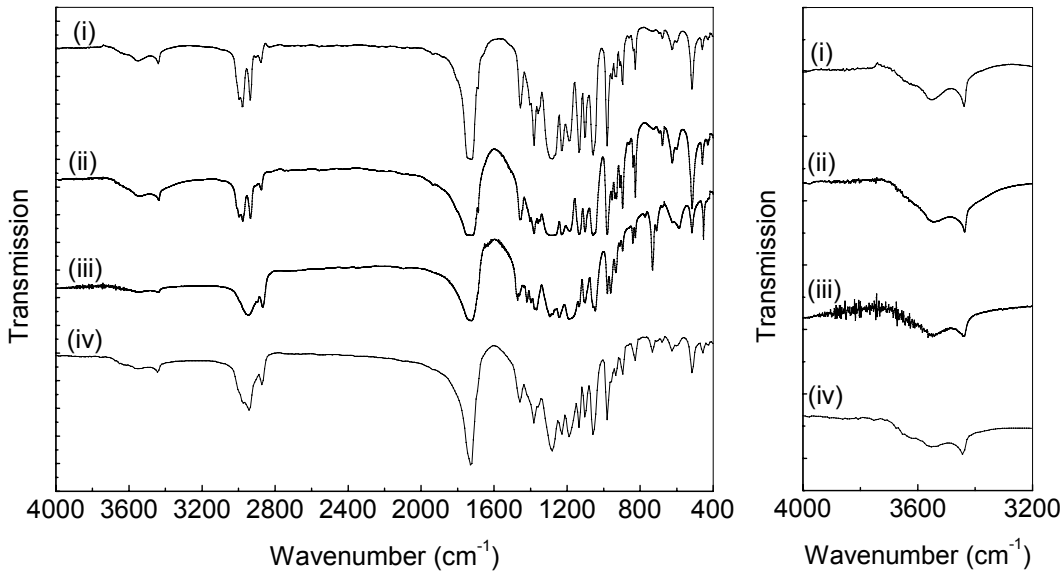


Figure 3.5. IR spectra of different polymers: (i) PHB-diol (M); (ii) PHB-diol (P); (iii) Poly[HB(56wt%)-*co*-CL(44wt%)] (sample B in Table 3.1); (iv) Poly[HB(28wt%)-*co*-CL(72wt%)] (sample D in Table 3.2).



(PCL-PHB-PCL) *tri*-block structure, the OH end groups would be the same, thus giving only one absorption peak in the IR spectrum.

The weight percentage of PHB and PCL block in the co-polymer was calculated using the repeating unit *n* and *p* for PHB and PCL block, respectively, obtained from NMR analysis. In the case of the polymer prepared above, *n* is 23.5, thus the molecular weight for PHB block is 86 x (23.5+1) + 1 + 60 = 2168; p is 14.2, therefore, the molecular weight for PCL block is 114 x (14.2 +1) + 1 = 1734. Based on these results, it can be deduced that the *di*-block polymer contains 56wt% of PHB block and 44wt% of PCL block. This method was used to calculate the weight ratio of the two blocks for all *di*-block polymers in this study.

Enzymatic ring-opening polymerization of ε-caprolactone with PHB-diol(P):

PHB-diol(P). For the easy investigation of the selectivities of the primary and secondary OH group of PHB-diol in the enzymatic ROP and for the easy preparation and characterization of the corresponding *di*-block copolymer with simpler structure, PHB-diol that contains 100% structure A with a primary and a secondary OH end groups was designed as the initiator. This type of PHB-diols with M_n between 2000-4000 g/mol was prepared by transesterification of PHB and ethylene glycol according to the same procedure for the preparation of PHB-diol(M),[82] but with large excess of ethylene glycol to PHB. The reaction conditions and results were summarized in Table 3.2. As shown in Table 3.2, catalyst amount was found to be an important factor to control the molecular

Table 3.2 Preparation of PHB-diol (P) *via* transesterification of PHB with ethylene glycol.

Code	PHB	EG	Cat	Diglyme	Temp	Time	M_n (GPC)	M_w (GPC)	M_w / M_n	T_m	T_g
	g	ml	mg	ml	°C	h	g/mol	g/mol		°C	°C
P1	0.4	2.0	1.2	1.6	135	4	4800	7900	1.66	154	
P2	0.4	2.0	2.4	1.6	135	4	3700	5300	1.42	146 / 153	
P3	0.4	2.0	4.8	1.6	135	4	3500	4800	1.37	133 / 147	-7.7
P4	0.4	2.0	7.2	1.6	135	4	3400	4700	1.38	133 / 146	-9.3
P5	0.4	2.0	9.6	1.6	135	4	3400	4700	1.38	128 / 143	-6.5
P6	8.0	40	11	32	135	4	3700	5100	1.38	134 / 149	-5.0
P7	0.5	0.3	12	2.0	135	4	3200	4500	1.41	148 / 153	
P8	10	15	20	40	135	4	2300	2700	1.17	115 / 129	-

EG: Ethylene Glycol Cat. : Dibutyltin dilaurate

weight of PHB-diol(P). With higher catalyst amount applied in the tranesterification, a lower molecular weight of PHB-diol(P) was obtained. Their desired chemical structures were confirmed by ^{1}H-NMR analysis. As an example, the ^{1}H-NMR spectrum of PHB-diol(P) with M_n of 3700g/mol (GPC) was shown in Figure 3.6(i). The primary OH and secondary OH group absorbed at 4.58 ppm (*c* proton) and 4.45 ppm (*d* proton), respectively, with nearly equal intensities. Similar to the case of PHB-diol(M), the molar ratio of structures A and B in PHB-diol(P) was estimated based on the intensities of *h* proton from the primary OH end and *u* proton from the secondary OH end as 100:0. The number of repeating unit n was calculated as 21.7 according to the ratio of *m*/*u* absorption intensity, which gave a M_n of 2010g/mol for PHB-diol(P). The primary and secondary OH groups of PHB-diol(P) showed two different absorptions at 3437 cm^{-1} and 3538 cm^{-1} in the IR spectrum in Figure 3.5(ii).

The physical properties of the new type of PHB-diols were characterized by DSC, and the T_m and T_g were found to be dependent on the M_n: PHB-diol (P) with M_n of 3700g/mol (GPC; M_w/M_n of 1.38) has T_m of 134 and 149^{o}C and T_g of -5^{o}C, while PHB-diol (P6) with M_n of 2300g/mol (GPC; M_w/M_n of 1.17) has T_m of 115 and 129^{o}C and T_g of -11.9^{o}C. Both of them are good hard segment for block co-polymer syntheses. For the preparation of biomaterials with the possibility of sterilization, T_m of the polymers needs to be above 120^{o}C. Therefore, PHB-diol(P) is an appropriate hard block for the preparation of such type of materials.

Ring-opening polymerization with PHB-diol(P). New batches of lipase-catalyzed ROP of CL with PHB-diol(P) (M_n of 3700, GPC) as the initiator were carried out in dioxane (experiments 12-15 in Table 3.3). The optimal conditions established for PHB-diol(M) such as 70 ^oC, CL:E in 8:1 wt ratio, and CL:Sol in 1:3 wt ratio were used for the ROP. Reaction for 16 h with a molar ratio of CL:PHB-diol(P) in 50:1 gave a block co-polymer with M_n (GPC) of 5500 g/mol. The molecular weight achieved here is similar to that obtained in the ROP with PHB-diol(M). Further increase of the molar ratio of CL:PHB-diol(P) to 100:1 did not increase the M_n of the final polymer. Changing the CL:E ratio from 8:1 to 4:1 did not result in big change of the polymer molecular weight, either. In experiment 16, ROP of CL and PHB-diol(P6) with M_n(GPC) of 2300g/mol was carried out at similar conditions, affording a polymer with M_n (GPC) of 5500g/mol. To further increase the molecular weight of the polymer, toluene was examined as solvent (experiments 17-21). The ROP of CL with PHB-diol(P) were initially performed at the optimal conditions established for dioxane: reaction with CL and PHB-diol(P) in 100:1 and 50:1 molar ratio, respectively, gave the corresponding polymers with M_n of 8900 and 7900 g/mol, respectively. The reaction conditions were also varied in other experiments, which did not improve the molecular weight of the polymer. Nevertheless, the polymers prepared in toluene have higher M_n than those prepared in dioxane. This is possibly due to the different log P value of the solvents. Toluene with log P of 2.5 is possibly less toxic to the enzyme than 1,4-dioxane with log P of -0.42. All the polymers were isolated in 69%-90% yield with 64-88% CL conversion by using the same method descript for the ROP with PHB-diol (M).

Figure 3.6. ^{1}H-NMR spectra in $DMSO_{d6}$ at 333K: (i) PHB-diol(P); (ii) Poly[HB(28wt%)-*co*-CL(72wt%)] (sample D in Table 3.3).

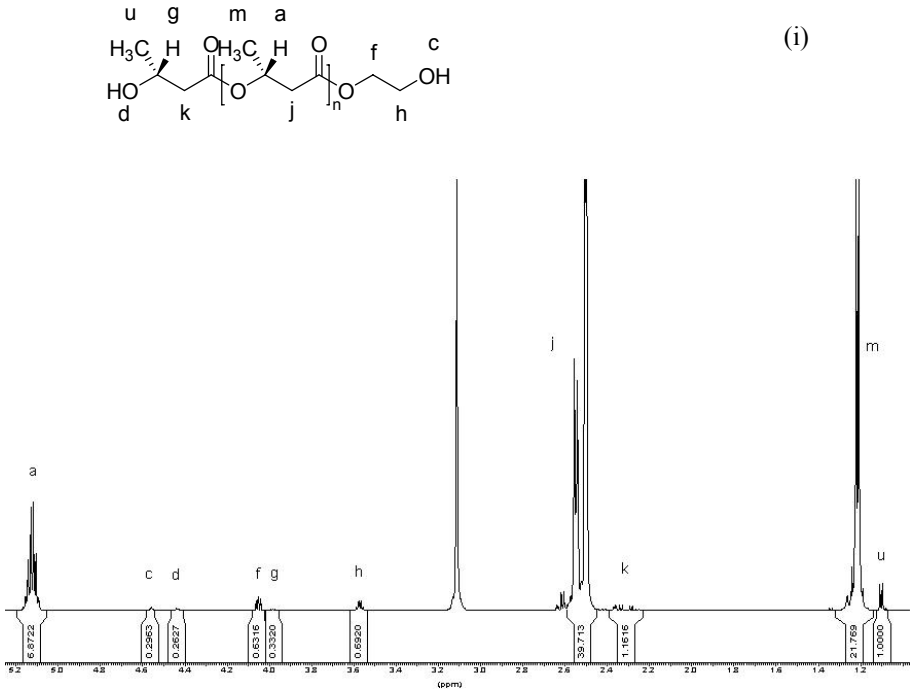


Table 3.3. Ring-opening polymerization of ε-caprolactone with PHB-diol (P) catalyzed by Novozym 435 in dioxane or toluene at 70 °C.

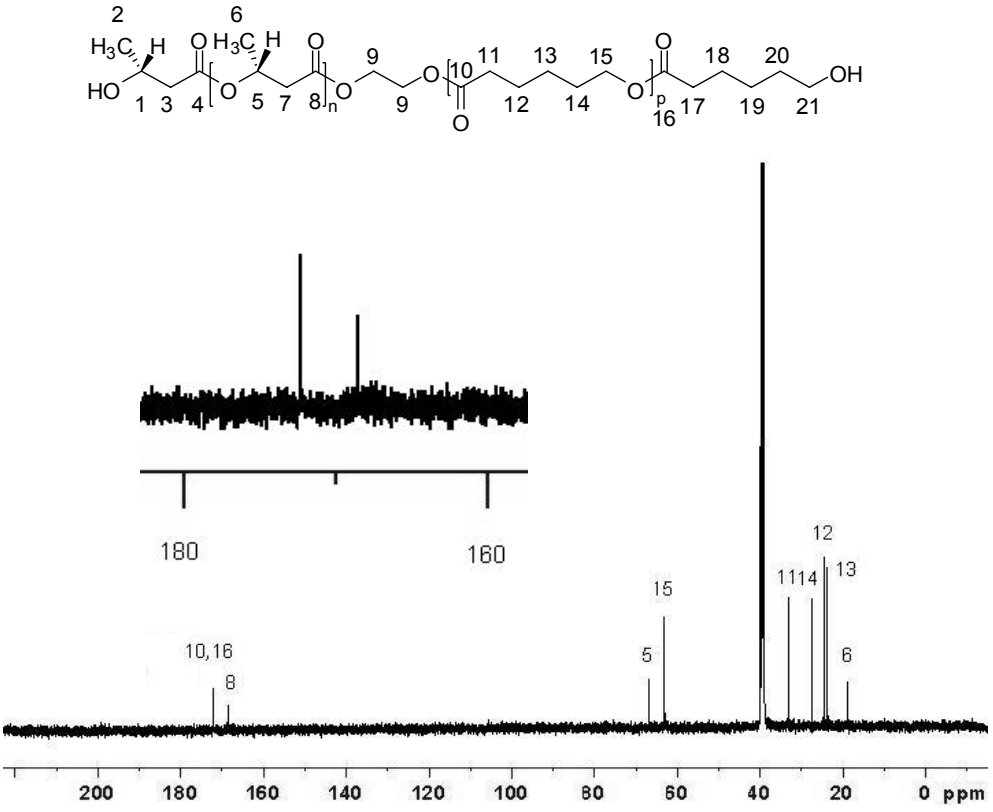
No.	Sol[a]	CL:**E**[b]	CL:PHB-diol	CL:**Sol**	Time	M_n (GPC)	M_w/M_n	Yield	CL Conv.	Diblock	T_m	T_g	Code
		wt:wt	mol:mol	wt:wt	h	g/mol		%	%	%	°C	°C	
12	D[c]	8:1	100:1	1:3	16	5300	1.56	73	69		51.3 / 132.3 / 148.3	-57.5	
13	D	8:1	50:1	1:3	16	5500	1.55	79	73		52.0 / 135.7 / 150.0	-57.7	
14	D	4:1	100:1	1:3	12	5200	1.75	88	86		50.0 / 128.7 / 145.7	-60.0	
15	D	4:1	50:1	1:3	48	4200	1.57	69	64	100	48.3 / 123.3 / 141.0	-59.1	
16	D	4:1	75:1	1:3	48	5500	1.75	76	73	97	48.7 / 99.0 / 116.0	-55.4	
17	T[d]	8:1	100:1	1:3	16	8900	1.93	89	87		56.7 / 154.3	-57.3	
18	T	8:1	50:1	1:3	16	7900	1.90	86	82	100	50.0 / 147.0	-60.0	D
19	T	4:1	100:1	1:3	12	7900	1.86	90	88		50.0 / 153.3	-58.8	
20	T	16:3	100:1	1:4	8	7900	1.98	87	85		52.0 / 153.3	-60.3	
21	T	16:3	100:1	1:6	8	5600	1.95	79	76	98	54.0 / 149.0	-61.0	E

a: **Sol** for solvent, Dioxane. b: **E** for immobilized enzyme, Novozym 435. c: D for dioxane. d: T for toluene. e: P8 for PHB-diol (P8) with M_n (GPC) of 2300 g/mol.

Structure Analysis. Polymers from experiments 15, 16, 18, and 21 were analyzed by ^{1}H-NMR and they all contain *di*-block poly(HB-*co*-CL) in 97%-100%. The ^{1}H-NMR spectrum of sample D (experiment 18) from Table 3.3 was given in Figure 3.6(ii). The *di*-block structure was clearly evidenced by the absorption of u proton at the end of PHB block and the *zz*, *ww*, *yy* protons at the end of PCL block. The intensity ratio of *ww* and *yy* signals (4 protons,1.45 ppm) and *u* signal (3 protons, 1.10 ppm) was 1.26:1, which corresponds to a ratio of the two ends of the polymers about 0.97:1. New signal of *e* proton appeared at 4.22 ppm, indicating the formation of polymer. From the ^{1}H-NMR spectrum, the ratio of *m/u* signal intensities of the polymer was determined as 21.7 which is exactly the same as that for PHB-diol(P). As previously described, the formation of *tri*-block polymer would increase the ratio of *m/u*. Therefore, it can be concluded that there is no *tri*-block structures in the polymer sample D. The ratio of the absorption intensities of *z* proton in PCL block and *a* proton in the PHB block was determined as 4.02:1, thus the *p* in the PCL bock can be deduced as 43.6. This led to the establishment of the polymer M_n as 7100g/mol. Calculation based on the intensities of *m* and *x* protons gave a M_n of 6900g/mol for the polymer. These values are in good correlation to the M_n measured by GPC (7900g/mol).

The ^{13}C NMR spectrum of sample D in Table 3.3 was shown in Figure 3.7. In the area of 160-180 ppm, the two signals observed are the carbonyl groups of the PHB and PCL block. No other carbonyl signals were detected, suggesting no random copolymers formed during ROP. The *di*-block structure was further confirmed by two different absorption of the primary and the secondary end OH groups at 3438 cm^{-1} and 3533 cm^{-1} in the IR spectrum of sample D shown in Figure 3.5(iv). Based on NMR analysis, n =

Figure 3.7. ^{13}C-NMR spectrum of poly[HB(28wt%)-*co*-CL(72wt%)] (sample D in Table 3.3) in $DMSO_{d6}$ at 333K.



21.7 and p = 43.6, thus the ratio of PHB and PCL block was established as 28/72 (wt/wt) for the polymer.

Mechanism consideration of ring-opening polymerization of *ε*-caprolactone and PHB-diol.

Is the polymerization really initiated by the PHB-diol? Is it possible that PCL is first formed by water-initiated ring-opening polymerization and then it reacts with PHB-diol *via* transesterification to give the co-polymer? To answer these questions, control experiments were carried out in 1,4-dioxane with CL and novozym 435 at 70°C and at the same ratio of CL/enzyme/solvent as that used for the synthesis of sample B in Table 3.1. Reaction for 16 h without pre-drying gave PCL with M_n of 12000g/mol (GPC), while reaction under anhydrous conditions for 16 h afforded a PCL with M_n of 5000g/mol (GPC). From these experiments, the possible formation of PCL in our polymerization reactions can not be excluded. Based on the known mechanism, even only a very small amount of water could initiate the polymerization to give PCL. Further control reaction was to examine the possible transesterification between PHB-diol and PLC. Reaction of PHB-diol(M) (M_n of 3000 g/mol) and PCL (M_n of 5000 g/mol) in 1:1 molar ratio with Novozym 435 as catalyst was performed in dioxane under the anhydrous conditions. The M_n of the reaction mixture was 4400g/mol (GPC) at the beginning and dropped to 2200g/mol (GPC) at 48 h. The product was isolated by the same procedure used for ROP and analyzed by ^{1}H- and ^{13}C-NMR. The *m/u* signal ratio of the product in the ^{1}H-NMR spectrum was drop to 16.9 from 23.6 of PHB-diol(M), suggesting the degradation of PHB-diol. This must be caused by the Novozyme 435-catalyzed transesterification

between PCL and PHB-diol, since no such degradation was observed in the same system without enzyme. In the ^{13}C-NMR, in addition to the signals at 168 and 172 ppm of the carbonyl group of the PHB and PCL block, two signals were observed at 169 and 171 ppm. These were the absorptions of the different type of carbonyl groups of random co-polymer. Thus, a mechanism of formation of PCL followed by transesterification with PHB-diol would generate co-polymers with decreased M_n, decreased *m*/*u* signal ratio in ^{1}H- NMR, and new signals in ^{13}C-NMR spectrum for random polymer units. Since all these phenomena were not observed in our polymer preparation, we can exclude such mechanism for our reaction.

The mechanism of enzyme-catalyzed ROP of CL was well known. Accordingly, the steps of our ROP were proposed in Scheme 3.2. The first step is the opening of CL with lipase to give an acyl-enzyme intermediate (EM). Afterwards, the intermediate is reacted with an OH group of PHB-diol to form new molecule M and release the lipase. As the primary OH group is more reactive and has less stereo-hindrance than the secondary OH group, it is preferentially reacted with lipase to give compound M with a secondary OH end group from PHB block and a primary OH end group from CL. While the first two steps can be repeated, the compound MH could also react with EM to prolong the chain and release lipase. Also in this step, the primary OH group of CL/PCL in compound MH should be more reactive than the secondary OH group of PHB block, thus generating a *di*-block polymer with a secondary OH group at end of PHB block and a primary OH group at the end of PCL block. Although we can not exclude the possibility of initiating ROP of CL by the trace amount of water in the system such as in the enzyme hydration shell, the primary OH group of PHB-diol should be more reactive than water,

thus effectively suppressing the water-initiated ROP. Even if PCL monomer and oligomer could be formed by water-initiated ROP, they could not react with the existing PHB-diol, its derivatives M and MH, and Poly(HB-*co*-CL) *via* enzymatic transesterification, since the enzyme active center is occupied through the formation of EM with CL in large excess. The only possibility remained for PCL monomer and oligomer is to react with EM for the elongation of the PCL chain, which is in competition with the elongation of poly(HB-*co*-CL). In fact, only very small amount of PCL monomer and oligomer was observed in our experiments, and these side products were easily removed by precipitating Poly(HB-*co*-CL) in chloroform/*n*-hexane (1:9).

Physical properties of *di*-block poly[(*R*)-3-hydroxybutyrate-*co*-ε-caprolactone]s

The melting temperature (T_m) and glass transition temperature (T_g) of several poly(HB-*co*-CL)s, PCL, and PHB-diols were measured by DSC. The data were summarized in Table 3.3 and 3.4. As shown in Figure 3.8, T_m and T_g were determined from the second heating curve. T_m of PHB-diol(P) was found to be 134°C and 149°C, and T_m of PCL with a M_n(GPC) of 12000 g/mol was 57°C. Poly[HB(55wt%)-*co*-CL(45wt%)] (sample E) and poly[HB(28wt%)-*co*-CL(72wt%)] (sample D) showed T_m from both PHB and PCL blocks, with the values of 147-149 °C and 50-54°C. T_m of the PHB block is a broad peak in DSC, and in some case it is split into two peaks. T_g of PHB-diol(P) and the PCL was determined to be -5°C and -63°C, respectively. The polymers D and E showed a T_g of -60°C and -61°C, respectively, which was obviously the T_g from the PCL block.

Scheme 3.2 Mechanism of enzyme-catalyzed ring-opening polymerization of ε-caprolactone with PHB-diol as initiator.

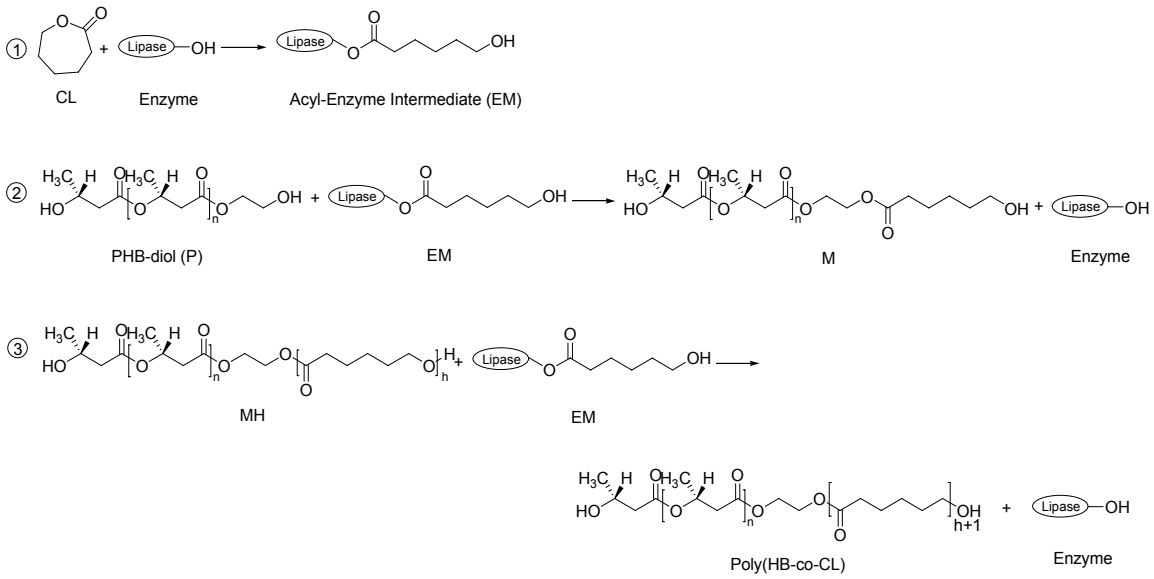
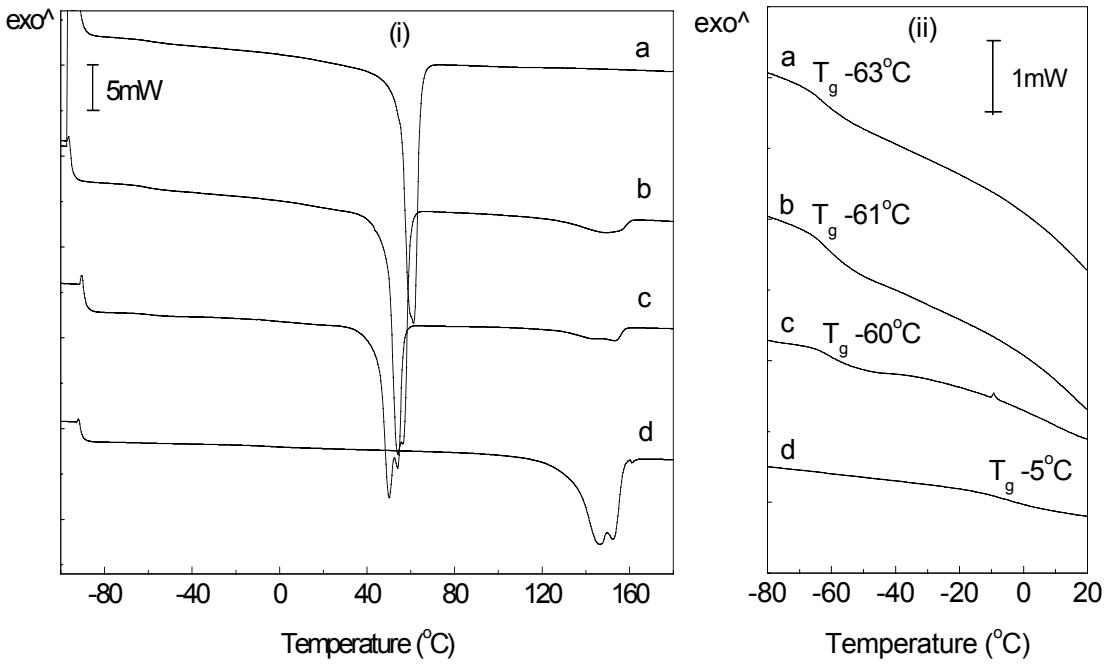


Table 3.4. Physical properties of selected poly(HB-*co*-CL)s, PHB-diol and PCL

Polymer	Code	Initiator	M_n (GPC)	M_n (NMR)	HB:CL[a]	T_m	T_g
			g/mol	g/mol	wt%	°C	°C
PHB-diol(M)			3000	2380		137 / 147	-4
PHB-diol(P)			3700	2010		134 / 149	-5
Poly(HB-*co*-CL)	A	PHB-diol(M)	6700	6100	32:68	54 / 120 / 139	-57
Poly(HB-*co*-CL)	B	PHB-diol(M)	5400	3900	56:44	53 / 123 / 145	-57
Poly(HB-*co*-CL)	C	PHB-diol(M)	8400	7500	26:74	53 / 121 / 140	-56
Poly(HB-*co*-CL)	D	PHB-diol(P)	7900	7100	28:72	50 / 147	-60
Poly(HB-*co*-CL)	E	PHB-diol(P)	5600	3400	55:45	54 / 149	-61
PCL		water	12000			57	-63

a: The ratio was calculated based on NMR analysis

Figure 3.8. DSC spectra of (a) PCL with M_n 12000 (GPC), (b) Poly[HB(28wt%)-*co*-CL(72wt%)] (sample D in Table 3.3), (c) Poly[HB(55wt%)-*co*-CL(45wt%)] (sample E in Table 3.3), and (d) PHB-diol(P): (i) The second heating curve; (ii) The enlarged second heating curve.



The T_g of PHB block was not detectable in all polymers prepared in this study. As shown in Table 3.4, all polymers containing 44-74wt% of PCL block have a T_g between -57°C and -61°C for the elastomeric domain. Thus, introduction of the PCL block significantly improved the thermal properties of block co-polymers for potential thermoplastic application.

In Figure 3.9, the DSC curves of different samples were compared, including PCL, PHB-diol(M), a mixture of PHB-diol(M) and PCL with weight ratio of 1:1, and poly[HB(56wt%)-*co*-CL(44wt%)] (sample B). While the 1:1 mixture demonstrated exactly the same T_m values as those of PHB-diol and PCL, the block co-poly(HB-*co*-CL) showed lower T_m than PHB-diol and PCL. This confirmed once again the formation of block-copolymer.

The crystallinity of the polymers was investigated by the WAXD. As shown in Figure 3.10, poly[HB(56wt%)-*co*-CL(44wt%)] (sample B) and poly[HB(26wt%)-*co*-CL(74wt%)] (sample C) showed very similar patterns to that of PHB-diol and PCL, but with decreased intensity: two major peaks from PCL at 2θ of 21.4^o and 2θ of 23.8^o and two major peaks from PHB-diol at 2θ of 13.6^o and 2θ of 17.0^o were founded in these copolymer. This indicated that the crystalline structures of the copolymers were similar to each of the two homopolymers. Thus, the polymer should show T_m from both PHB and PCL blocks. The crystallinities of poly(HB-*co*-CL)s, PHB-diol, PCL were estimated based on the ratios of crystalline peak area and amorphous peak area by using the affiliated software of SHIMADZU 6000 X-ray diffractometer. While PHB-diol and PCL showed crystallinities of 21% and 26% respectively, the two *di*-block poly(HB-*co*-CL)s sample B and C demonstrated a slightly decreased crystallinity of 19%.

Figure 3.9. DSC spectra of different polymers: (i) PCL with M_n of 5000; (ii) Poly[HB(56wt%)-*co*-CL(44wt%)] (sample B in Table 3.1); (iii) Mixture of PHB-diol(M) and PCL with M_n of 5000 (GPC); (iv) PHB-diol(M).

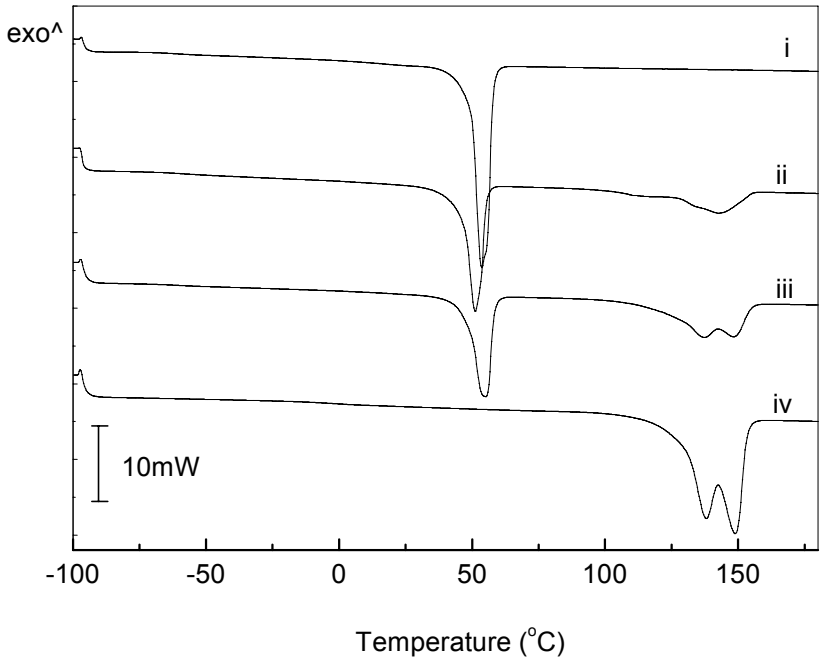
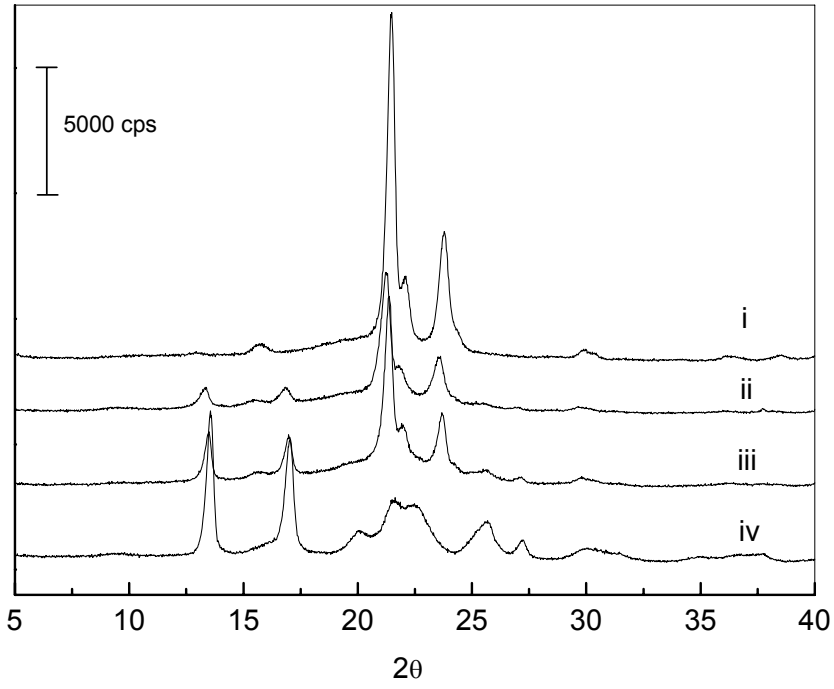


Figure 3.10. WAXD spectra of different polymers: (i) PCL with M_n of 12000 (GPC); (ii) Poly[HB(26%)-*co*-CL(74%)] (sample C in Table 3.1); (iii) Poly[HB(56wt%)-*co*-CL(44wt%)] (sample B in Table 3.1); (iv) PHB-diol(M).



3.4 Conclusion

Novel *di*-block co-polyesters containing PHB and PCL blocks were synthesized in high yield, for the first time, by enzymatic ring-opening polymerization of ε-caprolactone with PHB-diol. The structures of the *di*-block polymers with two different OH end groups were established by IR, ^{1}H-NMR and ^{13}C-NMR analyses. Poly(HB-*co*-CL)s with 44-74%(w/w) PCL demonstrated good thermal properties with T_g of about -60°C and T_m of 120-149°C and 50-54°C, being potentially useful thermoplastic biomaterials. Incorporation of PCL into the PHB-derived polyesters significantly improved the T_g of the materials.

Low molecular weight PHB-diol with a primary and a secondary OH end group was proven to be very useful for highly selective ring-opening polymerization, being the first example of using a telechelic macro-diol containing ester groups as an initiator. The primary OH end group of PHB-diol was found to initiate the ring-opening polymerization of ε-caprolactone, while the secondary OH end group was not reacted thus remaining as an end group of the final polymer. No enzymatic transesterification of PHB-diol and PCL happened and no random co-polymers formed during the polymerization. Optimal enzymatic polymerization conditions were established for the preparation of block co-polymers with different block ratio. This type of novel and selective ring-opening polymerization provides with new synthetic methods for preparing novel *di*-block co-polymers with functional end groups which could also be modified for other applications.

CHAPTER 4

CHEMO-ENZYMATIC PREPARATION OF THERMALPLASTIC BLOCK COPOLYMERS CONTAINING POLY[(*R*)-3-HYDROXYBUTYRATE] AND POLY(TRIMETHYLENE CARBONATE) BLOCKS

4.1 Introduction

Poly[(*R*)-3-hydroxybutyrate] (PHB) is a well-known biodegradable and biocompatible polymer synthesized by microorganisms and currently produced at >100 tones per year.[246] PHB has Young's modulus (E) of 1-4 GPa, elongation at break (ε_b) of <1%, melting temperature (T_m) of 175 ^oC, and glass transition temperature (T_g) of -4 ^oC,[1,2,247-249] thus being hard and brittle material. Although PHB has potential applications in several environmental and biomedical fields,[1,250-254] it can not be directly used as a thermoplastic biomaterial. However, the thermoplastic properties can be improved by preparing PHB-based block co-polymers containing appropriate soft blocks. For instance, poly(ε-caprolactone) (PCL) with E of 216 MPa, ε_b of 746 %, T_m of 60 ^oC, and T_g of -65 ^oC[255] was used as soft block to prepare PHB-based block-co-polymers[83,256] with good thermoplastic properties; microbial poly[(*R*)-3-hydroxyoctanoate] (PHO), a weakly crystalline and soft sticky material with T_m of 61 ^oC and T_g of -30 ^oC,[257-261] was also incorporated as soft block in PHB-based block co-polyesterurethane[84] and block co-polyesters[85] with improved thermoplastic properties.[84] We are interested in the preparation of thermoplastic PHB-based block co-polymer for soft tissue engineering,[262-264] and thus a much softer and more elastic segment than PCL and PHO is required. A potential candidate is biodegradable and biocompatible poly(trimethylene carbonate) (PTMC),[152-154] which has E of 3-7 MPa, ε_b of 1000%, and T_g of -25^oC.[216-218] PTMC was incorporated into several block-copolymers by metal-catalyzed sequential polymerization.[218,264-266] Among them, *tri*-block copolymer poly(LLA-TMC-LLA) demonstrated good plastic properties but poor thermal properties.[218] Here we want to

explore and evaluate the incorporation of PTMC block into PHB to achieve excellent thermoplastic properties for soft tissue engineering. Meanwhile, we also want to develop enzymatic method for controlled synthesis and setting of novel PTMC-containing polymer blocks for adjusting the thermoplastic properties of the final block copolymers.

The use of enzyme for polymer synthesis has received increasingly attention due to the *non*-toxicity and high selectivity. For instance, lipase-catalyzed ring-opening polymerizations (ROP) has become a useful tool for the synthesis of polyesters and polycarbonates.[104,143,267] Enzymatic ROP of trimethylene carbonate (TMC) with water and lactone, respectively, gave PTMC[155,157-158] and random copolymers poly(ester-carbonate),[159-161] respectively. However, this method has not been used for the preparation of block copolymers containing polycarbonates such as PTMC block. We previously developed a novel method for the enzymatic preparation of *di*-block copolyester poly(HB-*co*-CL) *via* enzymatic ROP of ε-caprolactone with telechelic PHB-diol as initiaor.[236] Here we want to extend this synthetic methodology to introduce PTMC block into PHB by enzymatic ROP of TMC with PHB-diol. Moreover, other polyester macro-diol such as PCL-diol could also be used as initiator for enzymatic ROP of TMC to prepare the corresponding block co-poly(ester carbonate) as potential soft segment. In this paper, we report the novel enzymatic syntheses, structural analyses, and physical properties of block co-poly(ester-carbonates) poly(HB-*co*-TMC) and poly(TMC-*co*-CL-*co*-TMC), the further polymerization of these block *co*-polymers to prepare block *co*-polyurethanes containing PHB and PTMC blocks, and the characterization of physical and mechanic properties of the final thermoplastic polymers.

4.2 Experiments

Materials. Novozym 435 (immobilized *Candida Antarctica* lipase B, 10000 PLU/g) was purchased from Novozymes. Trimethylene Carbonate (TMC) (99%) was purchased from Boehringer Ingelheim, Germany. ε-Caprolactone(99%), 1,4-dioxane (99.8%), toluene (99.8%), methylene diphenyl 4,4'-diisocyanate (MDI) (98%), and *N*, *N*-dimethylformamide (DMF, 99%) were purchased from Aldrich. Chloroform (HPLC, 99.9%) and methanol (HPLC, 99.9%) were obtained from Tedia. Telechelic hydroxylated poly-[(*R*)-3-hydroxybutrate] (PHB-diol, M_n of 3000, GPC) was prepared according to the published procedures.[82, 236] Novozym 435 and PHB-diol were dried in vacuum oven at 40 °C for 12 h, 1,4-Dioxane and toluene were dried by refluxing over sodium/benzophenone under argon, and DMF was dried with CaH_2 for 24 h and freshly distilled before use.

Synthesis of block co-poly[HB (26 wt%)-*co*-TMC (74 wt%)] by enzymatic ring-opening polymerization of TMC with PHB-diol: To a pre-dried mixture of PHB-diol [66 mg; M_n of 2200 (NMR) and 3000 (GPC) g/mol], TMC (305 mg), and Novozym 435 (72 mg) in a schlenk tube containing a magnetic stirring bar was added under argon atmosphere freshly distilled toluene (1.2 mL). The polymerization was performed at 50°C for 8 h, and the reaction was terminated by the addition of chloroform (6 mL). Novozym 435 was then removed by filtration, and the filtrate was subjected to evaporation under reduced pressure to remove toluene and chloroform. The residue was dissolved in chloroform (2 mL) and treated with methanol (18 mL), and the product was precipitated

at 4 °C for 30 min. After filtration, the precipitates were dried under vacuum in a rotary evaporator and then in a vacuum oven at 40 °C for 24 h. This gave 323 mg (87% yield) of poly(HB-*co*-TMC) with a M_n of 8700 g/mol (GPC), a weight ratio of PHB/PTMC of 26/74, T_m of 154 °C, and T_g of -24 °C.

Synthesis of block poly[TMC-*co*-CL-*co*-TMC] containing 29 wt% PCL and 71 wt% PTMC by enzymatic ring-opening polymerization of TMC with PCL-diol: A mixture of Novozym 435 (400 mg), freshly distilled ε-caprolactone (20 g), and ethylene glycol (1.0 mL) in a dry schlenk tube were stirred under argon atmosphere at 70 °C for 8 h. After reaction, the mixture was treated with chloroform (8 mL) and stirred at r.t. for 30 min. Novozym 435 was filtered out, and chloroform was removed by evaporation under reduced pressure. The product was dissolved in chloroform/methanol (20 mL/180 mL) and precipitated at 4 °C for 30 min. The final product was collected by filtration and then dried in vacuum oven at 40 ^{o}C for 24 h, which gave 16.86 g (84% yield) of PCL-diol with M_n of 4200 g/mol (GPC), T_m of 46 °C, and T_g of -58 °C.

To a mixture of Novozym 435 (97 mg), PCL-diol prepared above (62 mg), and TMC (402 mg) in a schlenk tube was added freshly distilled toluene (1.6 mL) under argon atmosphere, and reaction was performed at 50 ^{o}C for 8 h. Work-up with the same procedure as that for the synthesis of poly(HB-*co*-TMC) gave 296 mg (64% yield) of poly(TMC-*co*-CL-*co*-TMC) with a M_n of 10600 g/mol (GPC), a weight ratio of PCL/ PTMC of 29/71, and T_g of -42°C.

Synthesis of polyurethane PU_{HBTMC} by polymerization of poly[HB(26 wt%)-*co*-TMC(74 wt%)] with MDI: To a solution of poly[HB(26 wt%)-*co*-TMC(74 wt%)] (1997 mg) in dry DMF (10 mL) in a schlenk tube containing a magnetic stirring bar was added a solution of MDI (104 mg) in DMF (5 mL) dropwise under Argon atmosphere. The mixture was stirred at 85 ^{o}C for 12 h. After cooling, methanol (60 mL) was added and the mixture was magnetically stirred for 30 min at room temperature. The product was precipitated, collected by filtration, washed with mixture of DMF/methanol (15 mL/60 mL), and dried in a vacuum oven at 50 ^{o}C for 24 h. 1982 mg of PU_{HBTMC} (94% yield) was obtained with a M_n of 53800 g/mol (GPC), T_m of 144 °C, T_g of -9 °C, E of 23 MPa, and ε_b of 252%.

Synthesis of polyurethane PU_{HBCL} by polymerization of poly[HB(28 wt%)-*co*-CL(72 wt%)] with MDI: Similar to the procedure for the preparation of PU_{HBTMC}, poly[HB(28 wt%)-*co*-CL(72 wt%)][43] (2002 mg) and MDI (90 mg) in DMF (15 mL) were reacted at 85 ^{o}C for 12 h, followed by the work-up and precipitation. 1914 mg of PU_{HBCL} (89% yield) was obtained with a M_n of 34600 g/mol (GPC), T_m of 143 and 46 ^{o}C, T_g of -48 °C, E of 838 MPa, and ε_b of 1.25 %.

Synthesis of polyurethane PU_{TMCCL} by polymerization of poly[TMC-*co*-CL-*co*-TMC] containing 38 wt% PCL and 62 wt% PTMC with MDI: Similar to the procedure for the preparation of PU_{HBTMC}, poly[TMC(62 wt%)-*co*-CL(38 wt%)] [M_n of 7600 g/mol (GPC); 2011 mg] and MDI (64 mg) in DMF (15 mL) were reacted at 85 ^{o}C for 12 h, followed by the work-up and precipitation. 1892 mg of PU_{TMCCL} (91% yield) was obtained with M_n of 41500 g/mol (GPC), E of 19 MPa, and ε_b of 62.35 %.

Synthesis of polyurethane $PU_{TMCCLHB}$ by polymerization of poly[TMC-*co*-CL-*co*-TMC] containing 38 wt% PCL and 62 wt% PTMC and PHB-diol with MDI: Poly[TMC(62 wt%)-*co*-CL(38 wt%)] [M_n of 7600 g/mol (GPC); 2012 mg], PHB-diol [M_n of 3000 g/mol (GPC); 62 mg], and MDI (72 mg) in DMF (15 mL) were reacted at 85 ^oC for 12 h, followed by the work-up and precipitation. 1906 mg of $PU_{TMCCLHB}$ (88% yield) was obtained with M_n of 57200 g/mol (GPC), E of 105 MPa, and ε_b of 37.80 %.

Gel permeation chromatography (GPC). Molecular weight analysis (M_n and polydispersity index M_w/M_n) was performed by using a Waters instrument, with Waters 510 pump, Waters 410 refractive index detector, and Waters HR4E, HR5E and HR6 columns placed in series. THF was used as the eluent for the measurement of PHB-diol and poly(HB-*co*-TMC)s at a flow rate of 1.0 mL/min and at 30°C. DMF was used as the eluent for the analysis of polyurethanes at a flow rate of 1.0 mL/min and at 35°C. Sample concentration was about 0.1% (w/v) and the injection volume was 100 μL. Polystyrene standards with molecular weights of 1310, 2970, 13900, 30200, 197000 and 696000 g/mol were used to generate a calibration curve.

Nuclear magnetic resonance (NMR). ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded with a Bruker AMX500 NMR instrument in $DMSO_{d6}$ at 333K. Chemical shifts were referred to TMS at 0 ppm.

Differential scanning calorimetry (DSC). The thermal properties of polymers were measured on a Mettler Toledo DSC 822 system. Nitrogen was used as purge gas with a flow rate of 20 mL/min. Samples of 10 mg were prepared in aluminum foils, where the aluminum weights of the sample and reference were closely matched. The samples were

heated from room temperature to 180 °C, cooled down to -100 °C, and heated again to 180 °C, all at a rate of 20°C/min. T_m and T_g of the samples were obtained from the second heating curves.

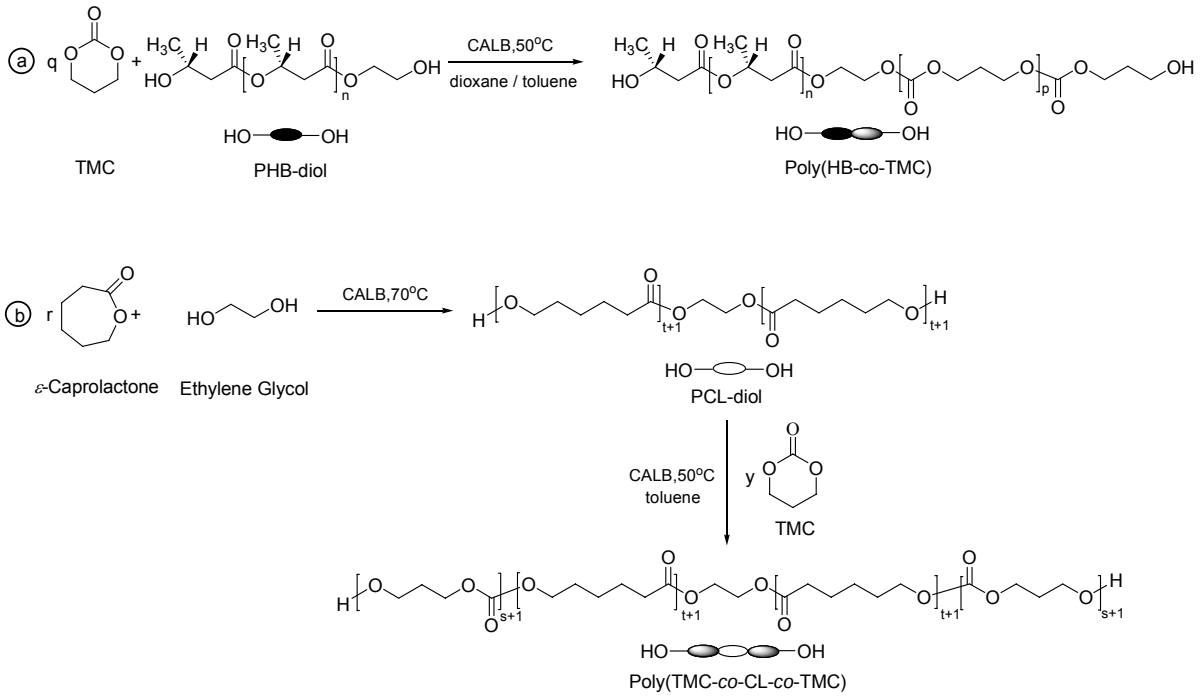
Fourier transform infrared spectrophotometer (FTIR). IR spectra of the polymers were analyzed with a SHIMADZU FTIR-8400 system using dimethylformamide (DMF) as solvent.

Tension test. Tension tests of polymer films were carried out on Instron 5569 double columns micro-force tester with a 10N load cell at a crosshead speed of 5 mm/sec at room temperature. The films were prepared as follows: 1.0 g of polymer was dissolved in 10 mL DMF, the solution was cast onto a PTFE plate, and the temperature was kept at 65 ^{o}C to remove DMF; the obtained film was further dried in a vacuum oven at 65 ^{o}C for 48 h; and finally, dog bone-shaped specimens [50 mm (length) × 3.25 mm (width) × 0.2 mm (thickness)] were prepared for tension tests.

4.3 Results and Discussion

Preparation of block poly(HB-*co*-TMC) by enzymatic ring-opening polymerization of TMC with PHB-diol.

To incorporate PTMC into PHB, Novozym 435 [immobilized *Candida antarctica* lipase B (CALB)]-catalyzed ring opening polymerization (ROP) of trimethylene carbonate (TMC) with telechelic hydroxylated poly-[(*R*)-3-hydroxybutyrate] (PHB-diol) as initiator was explored (Scheme 4.1. a). PHB-diol (M_n of 3000 g/mol, GPC) was prepared by transesterification of PHB with ethylene glycol according to the published procedures.[82, 236] 1,4-Dioxane and toluene with boiling point higher than 100 °C and good solubility for PHB-diol were examined as the solvent for the ROP. To avoid water-initiated ROP of TMC, both 1,4-dioxane and toluene were dried and freshly distilled before use, and the reactions were carried out at anhydrous conditions under argon atmosphere. The reaction temperatures were examined from room temperature to 70 °C, and different ratios of TMC/enzyme, TMC/PHB-diol, and TMC/solvent were explored to obtain best polymerization conditions as well as polymers with different ratio of PHB/PTMC blocks. Initial tests showed that the highest molecular weight of the product was achieved at 8 h, thus, a series of reactions were performed for 8 h. After the reaction, the reaction mixtures were treated with chloroform, the enzyme was removed through filtration, the solvent was removed, and the product was precipitated in chloroform/methanol (1:9). After drying under high vacuum at 50^{o}C for 24 h, the corresponding block copolymers poly(HB-*co*-TMC)s were obtained in 57-89% yield. The M_n of the polymers was determined by GPC as 4400-8700 g/mol. The reaction conditions, yields, and the molecular weights of the polymers are summarized in Table 4.1.



Scheme 4.1 Enzymatic preparation of PTMC, poly(HB-*co*-TMC), poly(TMC-*co*-CL-*co*-TMC) and poly(HB-*co*-CL-*co*-TMC) *via* ring-opening polymerization of TMC with water, PHB-diol, PCL-diol and poly(HB-*co*-CL) as initiator.

In entry 1-3 (Table 4.1), ratios of TMC/enzyme (E) from 2/1 to 4/1 were studied for the polymerization in dioxane at 50^{o}C at fixed ratios of TMC/PHB-diol and TMC/solvent (S). The molecular weight and yield of the resulting product increased with the increase of catalyst amount: M_n of 4700, 5000, and 5500 g/mol were obtained at ratio of TMC/E of 4/1, 3/1, and 2/1, respectively, after 8 h reaction. The increase of reaction time (entry 4 *vs* 1) from 8 h to 24 h resulted in a decreased molecular weight. To avoid the use of too much catalyst, the ratio of TMC/E of at 4:1 was used for further study on the polymerization. For entry 5 and 3, the reduction of ratio of TMC/PHB-diol from 100/1 to 75/1 resulted in slightly decrease of the polymer molecule weight.

Toluene was proved to be a much better solvent than dioxane for the polymerization. As shown in entry 3 and 6, M_n of the polymers increased from 4700 to 8700 g/mol by simple replacement of dioxane with toluene. Here again, the reduction of ratio of TMC/PHB-diol from 100/1 to 75/1 (entry 6 *vs* 7) or from 100/1 to 50/1 (entry 8 *vs* 9) resulted in the decrease of M_n of the polymer. This effect can be used to synthesize polymers with different molecule weight and thus different ratio of PHB/PTMC. Further increase of the ratio of TMC/enzyme from 4/1 to 6/1 gave rise to a significant decrease of polymer M_{n} (entry 6 *vs* 8). The temperature effects were examined from room temperature to 70 °C in entry 6, 10-13. The polymer molecular weight increased from r.t. to 50 ^{o}C, reached the maximum at 50-60 ^{o}C, and decreased from 60 ^{o}C to 70 ^{o}C. Thus, the best reaction temperature is 50 ^{o}C for the enzymatic ROP of TMC.

Table 4.1. Ring-opening polymerization of Trimethylene Carbonate with water, PCL-diol and PHB-diol catalyzed by Novozym 435 in dioxane and toluene.

Entry	Initiator	M_n [a] (g/mol)	M [b]: I [c]	M:E [d]	M:S [e]	Solvent	Temp. (°C)	Time (h)	M_n [f] (g/mol)	M_w/M_n	Yield (%)	Code
1	PHB-diol	2200	100:1	2:1	1:4	D [g]	50	8	5500	1.36	64	
2	PHB-diol	2200	100:1	3:1	1:4	D	50	8	5000	1.28	58	
3	PHB-diol	2200	100:1	4:1	1:4	D	50	8	4700	1.26	57	
4	PHB-diol	2200	100:1	2:1	1:4	D	50	24	4400	1.37	57	
5	PHB-diol	2200	75:1	4:1	1:4	D	50	8	4500	1.30	71	A
6	PHB-diol	2200	100:1	4:1	1:4	T [h]	50	8	8700	1.58	87	B
7	PHB-diol	2200	75:1	4:1	1:4	T	50	8	7400	1.59	89	
8	PHB-diol	2200	100:1	6:1	1:4	T	50	8	6600	1.57	84	
9	PHB-diol	2200	50:1	6:1	1:4	T	50	8	5400	1.65	85	C
10	PHB-diol	2200	100:1	4:1	1:4	T	40	8	7000	1.56	84	
11	PHB-diol	2200	100:1	4:1	1:4	T	60	8	8500	1.69	82	
12	PHB-diol	2200	100:1	4:1	1:4	T	70	8	7800	1.43	81	
13	PHB-diol	2200	100:1	4:1	1:4	T	r.t.	8	7100	1.45	84	D
14	PCL-diol	3000	100:1	4:1	1:4	T	50	8	9200	1.61	67	
15	PCL-diol	3000	200:1	4:1	1:4	T	50	8	10600	1.64	64	E
16	PCL-diol	3000	100:1	2:1	1:4	T	70	8	7700	1.58	55	F
17	PCL-diol	3000	200:1	4:1	1:4	T	70	8	8100	1.62	54	

a) Calculated from ^{1}H NMR; b) M: Monomer (TMC); c) I: Initiator, PHB-diol or PCL-diol; d) E: Enzyme, Novozym 435; e) S: Solvent; f) Measured by GPC; g) D: dioxane; h) T: toluene

Structural Analysis. From the ^{1}H NMR spectrum of PHB-diol (M_n of 3000 g/mol, GPC) shown in Figure 4.1(i), the number of monomer repeating unit *n* in PHB-diol was established as 24 based on the signal intensities of proton *m*/*u*. The M_n of PHB-diol was thus deduced as 2200 g/mol. In the ^{1}H NMR spectrum of poly(HB-*co*-TMC) (Sample B) shown in Figure 4.1 (ii), signals of protons *c*, *f*, and *h* of PHB-diol disappeared, and a new signal of proton *e* was observed. This indicates that the terminal primary OH group of PHB-diol was reacted with TMC to form the polymer. On the other hand, the ratio of signal intensity of proton *m*/*u* remained unchanged, and the signal of proton *d* was clearly observed. This suggests that the terminal secondary OH group of PHB-diol did not react with TMC and remained as the terminal group of the *di*-block polymer. Therefore, the ring-opening polymerization of TMC was initiated selectively with the primary OH terminal group of PHB-diol, which is similar to the ring-opening polymerization of *ε*-caprolactone with PHB-diol.[82] The unchanged ratio of proton *m*/*u* suggests also no degradation or transesterification of the PHB block during the reaction. The signals of protons of *aa*, *bb*, *cc*, and *dd* of PTMC block were easily assigned. Based on the signal ratio of *a*-proton and *aa*-proton of 7.8/38 (= *n*/2*p*), the number of TMC monomer repeating units *p* was deduced to be 58. Therefore, the molecular weight (M_n) of the block-copolymer was established as 8200 g/mol. This value is very close to the M_n of 8700 g/mol determined by GPC. Based on the M_n and structure established by NMR, the ratio of PHB and PTMC in the polymer can be deduced as 26/74 (wt/wt).

The analysis of ^{13}C NMR spectrum of poly(HB-*co*-TMC) (Sample B) in Figure 4.2 further confirmed that the polymer is a block copolymer rather than random copolymer: there were only two signals at 154 ppm and 169 ppm in the range of 150-170

Figure 4.1. ^{1}H NMR spectra of (i) PHB-diol and (ii) poly[HB(26 wt%)-*co*-TMC(74 wt%)] (Sample B, Table 4.1) in DMSO-$_{d6}$ at 333K

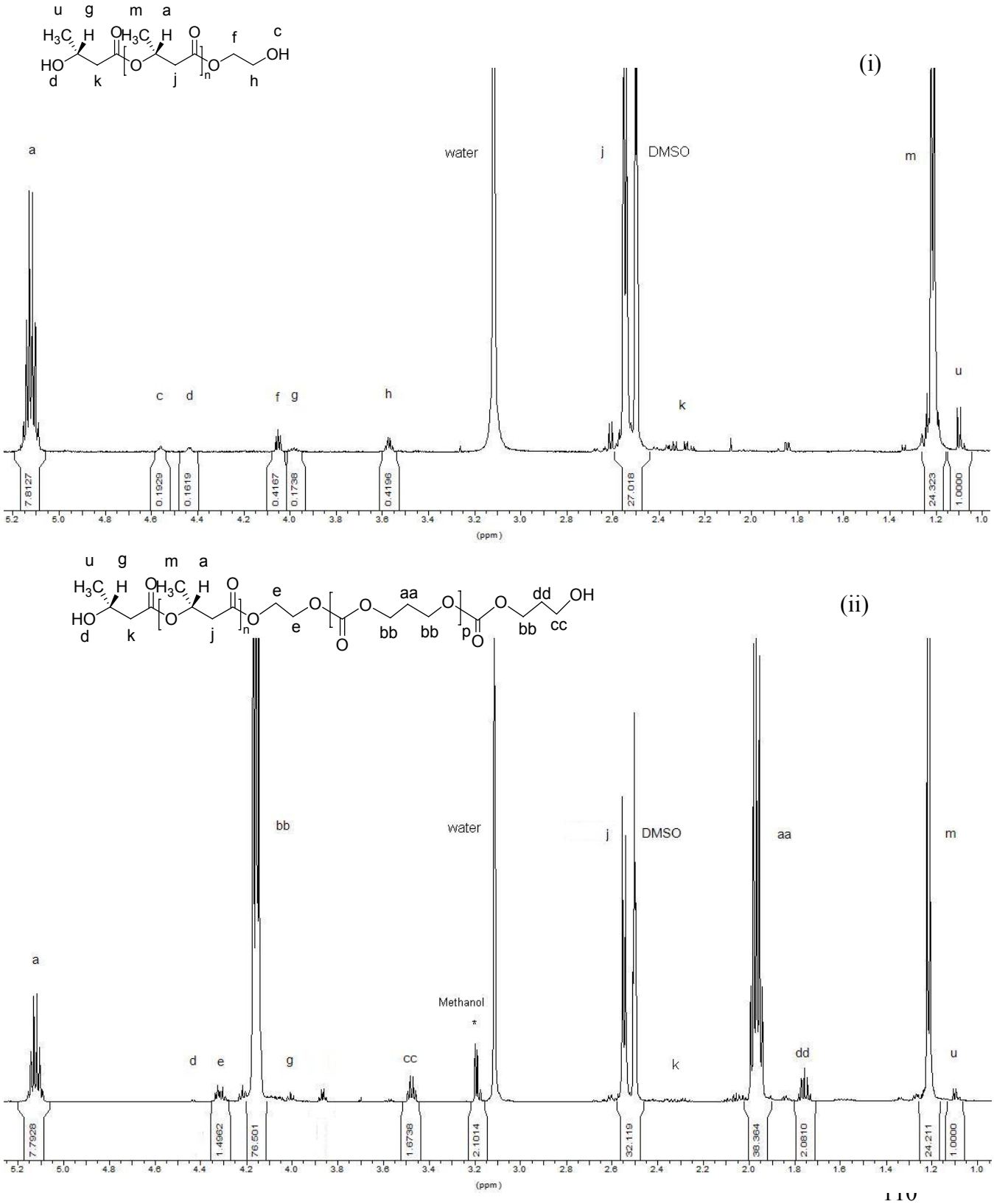
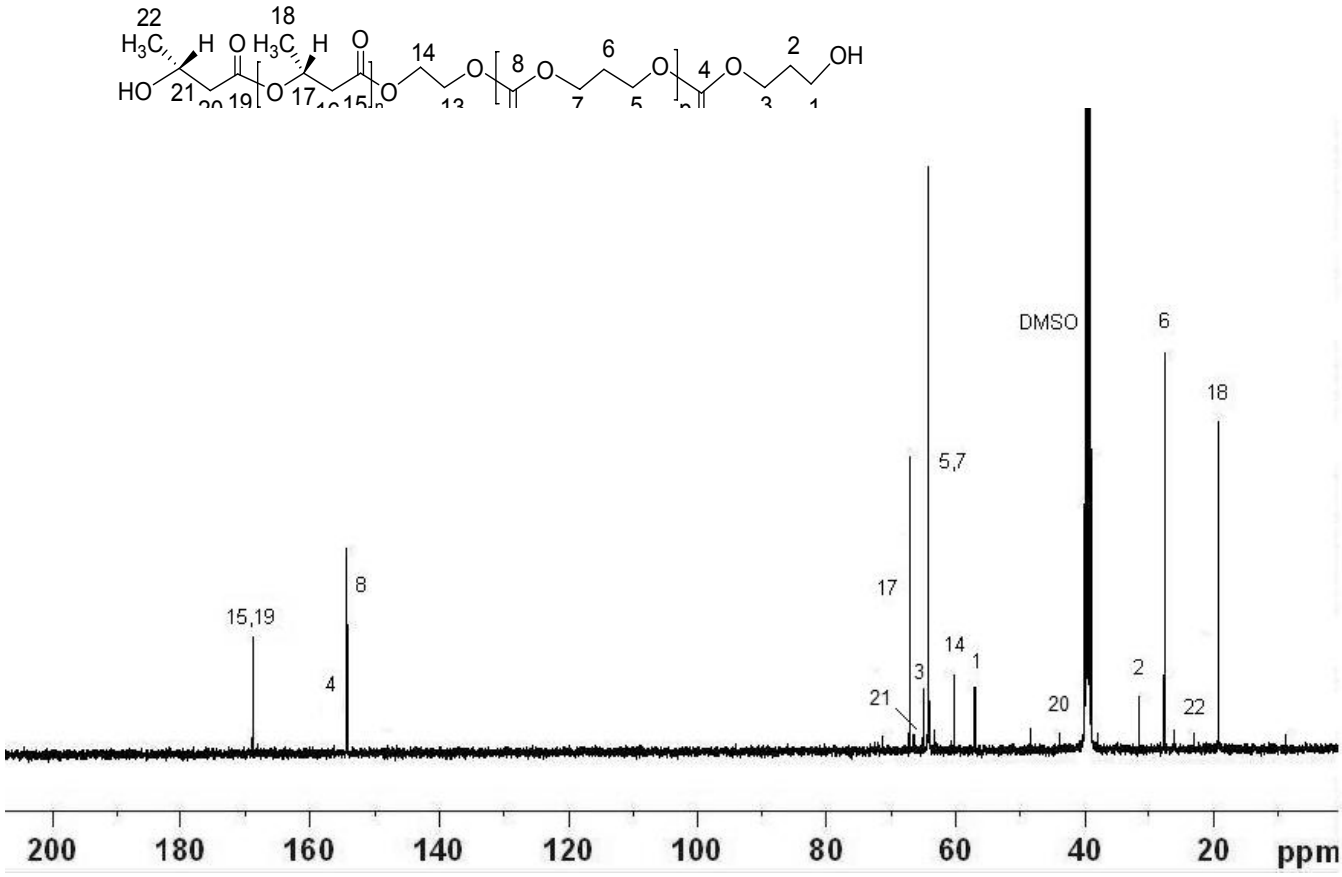


Figure 4.2. ^{13}C NMR spectrum of poly[HB(26 wt%)-*co*-TMC(74 wt%)] (Sample B, Table 4.1) in DMSO-$_{d6}$ at 333K

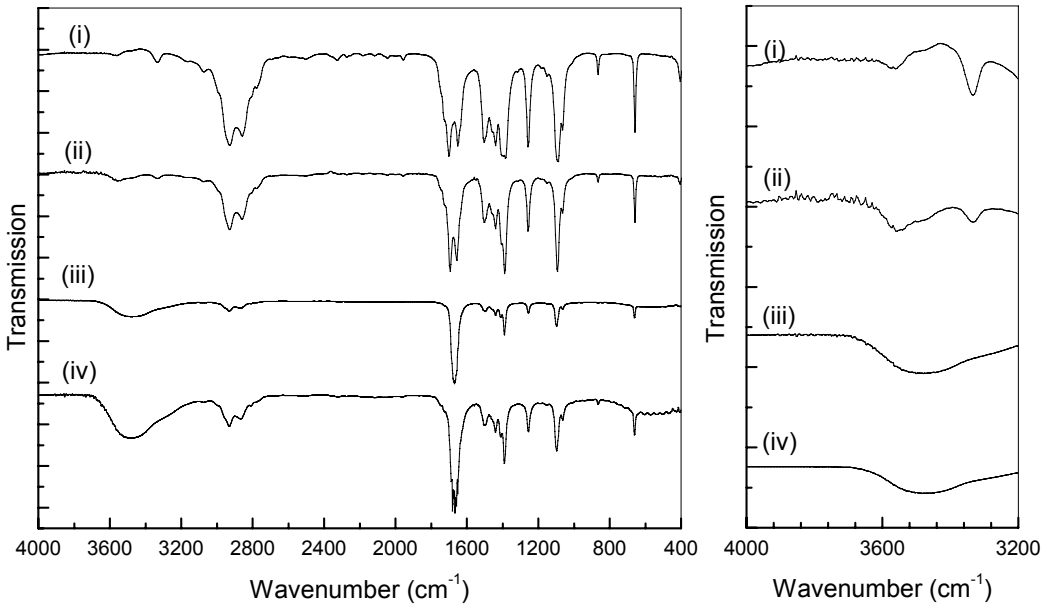


ppm, which were assigned to the carbonyl groups in PHB block and PTMC block, respectively; if random copolymer would be produced, there would be additional signals for the new type of carbonyl groups between 154-169 ppm. The *di*-block structure of poly(HB-*co*-TMC) (Sample B) with a primary and a secondary terminal OH groups was further evidenced in the IR spectrum in Figure 4.3 (ii): there were two absorption peaks at 3555cm^{-1} and 3332 cm^{-1} indicating the existence of two different types of OH groups; this is similar to the IR spectrum of PHB-diol in Figure 4.3(i) which showed two different absorptions at 3559cm^{-1} and 3332cm^{-1} for the primary and the secondary terminal OH groups, respectively; if the block-copolymer would have the structure of PTMC-PHB-PTMC, it would have the same type of terminal OH group at both ends and thus would give only one absorption peak in the IR spectrum.

Preparation of block poly(TMC-*co*-PCL) by enzymatic ring-opening polymerization of TMC with PCL-diol.

To explore the possibility of combining PTMC and PCL blocks as potential soft segment, block copolymer poly(CL-*co*-TMC)s were prepared. At first, PCL-diol with M_n of 3000 (^{1}H NMR) was prepared in 84% yield by Novozym 435-catalyzed ROP of ε-caprolactone with ethylene glycol as initiator at 70 °C without using any solvent (Scheme 4.1.b). PCL-diol was then used as initiator for enzymatic ROP of TMC to prepare poly(CL-*co*-TMC)s. The reactions were examined in toluene at different molar ratio of TMC/PCL-diol for 8 hours at 50 ^{o}C and 70 ^{o}C, respectively. The products were isolated using the same procedure for the preparation of poly(HB-*co*-TMC)s in 54-67% yield, with M_n (GPC) of 7700-10600 g/mol. As shown in entry 14 *vs* 15 and 16 *vs* 17 of Table 4.1, the increase of

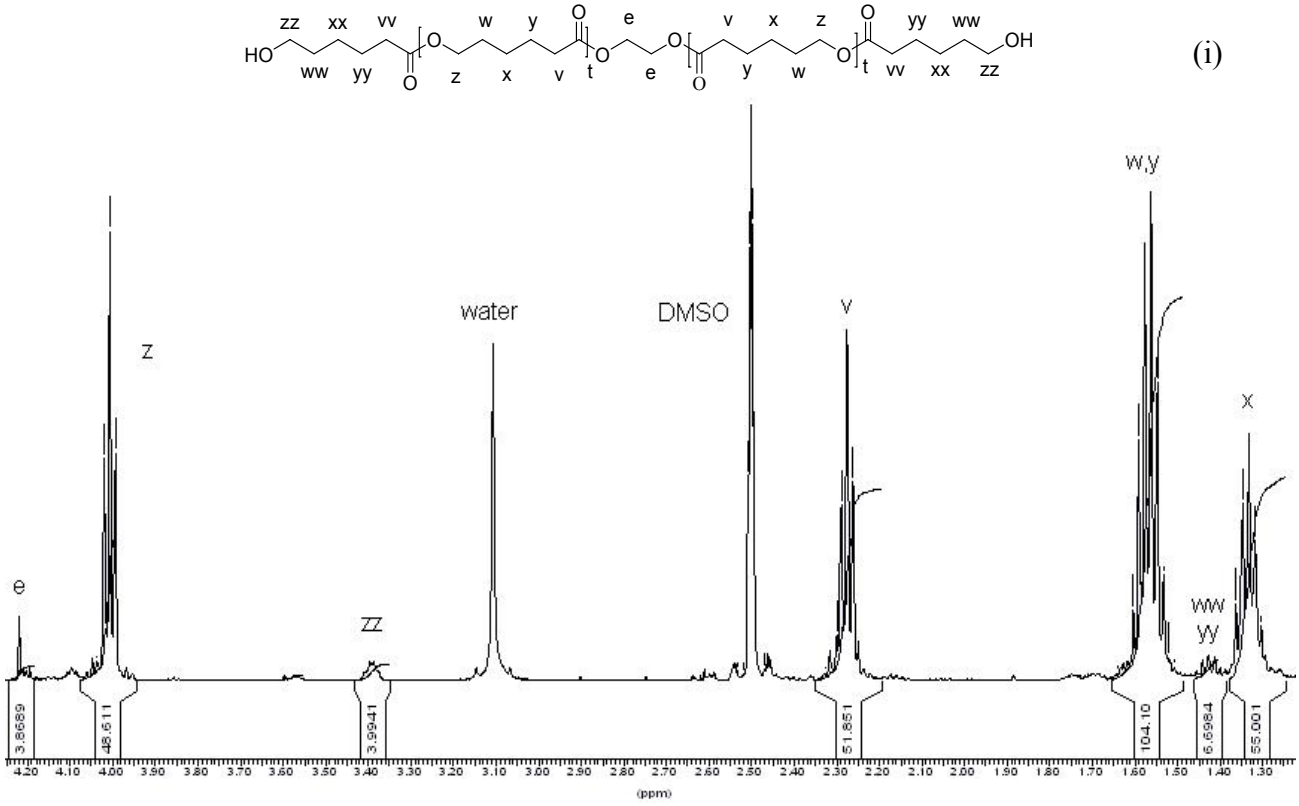
Figure 4.3. IR spectra of (i) PHB-diol; (ii) poly[HB(26 wt%)-*co*-TMC(74 wt%)] (Sample B, Table 4.1); (iii) PCL-diol; (iv) poly[TMC-*co*-CL-*co*-TMC] containing 29 wt% PCL and 71 wt% PTMC (Sample E, Table 4.1).



the ratio of TMC/PCL-diol increased the M_n of the final polymers at both temperatures. From entry 15 and 17 of Table 4.1, it can be seen that reaction temperature of 50 ^oC is better than 70 ^oC for producing higher molecular weight polymers. All effects of different parameters on the molecular weigh of the product in the enzymatic ROP of TMC with PCL-diol are similar to those of the ROP of TMC with PHB-diol described above.

Structural Analysis. The ^1H NMR spectrum of PCL-diol prepared above by enzymatic ROP of ε-caprolactone with ethylene glycol was shown in Figure 4.4 (i). No signals of protons from (-OC**H**$_2$-C**H**$_2$OH) were detected, and the signal of proton *e* was clearly observed. This suggested that both OH groups of ethylene glycol were reacted. The protons from the PCL backbone (*z*, *w*, *x*, *y*, *v*) as well as the protons from the terminals (*zz*, *ww*, *yy*) adsorbed at the expected areas. The signal intensity of proton *zz* is nearly the same as that of proton *e*, which confirmed the polymer structure shown in Figure 4.4 (i). Based on the signal ratio of proton *z*/*zz* of 12/1, the CL repeating t from the structure was deduced as 12. The M_n of PCL-diol was then established as 3000 g/mol. The ^1H NMR spectrum of the copolymer poly(CL-TMC) (sample E, Table 4.1) prepared from the PCL-diol was shown in Figure 4.4 (ii). The protons *aa* and *bb* of PTMC backbone and *z*, *y*, *x*, *w*, and *v* absorbed in the expected areas. Moreover, the signals for protons *zz*, *ww*, *yy* from PCL-diol ending group disappeared, and new signals for protons *cc* and *dd* of the ending groups of PTMC block were observed. This confirmed the structure of the polymer as *tri*-block poly(TMC-*co*-CL-*co*-TMC). This structure is the logical result for the ROP of TMC with PCL-diol which contains two equal terminal OH groups. Based on the signal ratio of proton *aa*/*v* of 2.77/1, the PTMC repeating unit *s* in the polymer

Figure 4.4. ^{1}H NMR spectra of (i) PCL-diol and (ii) poly[TMC-*co*-CL-*co*-TMC] containing 29 wt% PCL and 71 wt% PTMC (Sample E in Table 4.1) in DMSO-$_{d6}$ at 333K.



structure was deduced as 36. The M_n of poly(TMC-*co*-CL-*co*-TMC) was thus established as 10500, which is nearly the same as the value obtained by GPC analysis. The ratio of PCL and PTMC was thus established as 29/71 (wt/wt). In the ^{13}C NMR spectrum of the same polymer in Figure 4.5 there were only two signals at 154 ppm and 172 ppm which were assigned to the carbonyl groups of TMC and PCL blocks, respectively. This excluded the formation of random copolymer and thus confirmed the block copolymer structure.

Physical properties of block-*co*-poly(ester-carbonates).

The melting temperature (T_m) and glass transition temperature (T_g) of poly(HB-*co*-TMC)s, poly(TMC-*co*-CL-*co*-TMC)s, PHB-diol, and PTMC were measured by DSC. The data were summarized in Table 4.2, and the DSC curves were shown in Figure 4.6 (i). The *di*-block poly(HB-*co*-TMC)s with M_n of 4,500-8,700 g/mol (Sample A-D) showed the T_m of PHB block at 149-154 ^oC, which are slightly higher than the T_m of PHB-diol (143 °C). T_g of the *di*-block poly(HB-*co*-TMC)s were between -20 °C and -24 °C, which was obviously from PTMC block. T_g of PHB block was not detectable. Poly(TMC-*co*-CL-*co*-TMC)s with a M_n of 10500 (sample E) and 7700 g/mol (sample F / G) have no T_m, but a T_g of -42 ^oC and -48 ^oC, respectively. These values are higher than that of PCL-diol (-58 ^oC), but lower than that of PTMC (-27 ^oC). Incorporation of more PTMC into the polymers resulted in decrease of T_g.

Figure 4.5. ^{13}C NMR spectrum of poly[TMC-*co*-CL-*co*-TMC] containing 29 wt% PCL and 71 wt% PTMC (Sample E, Table 4.1) in DMSO-$_{d6}$ at 333K.

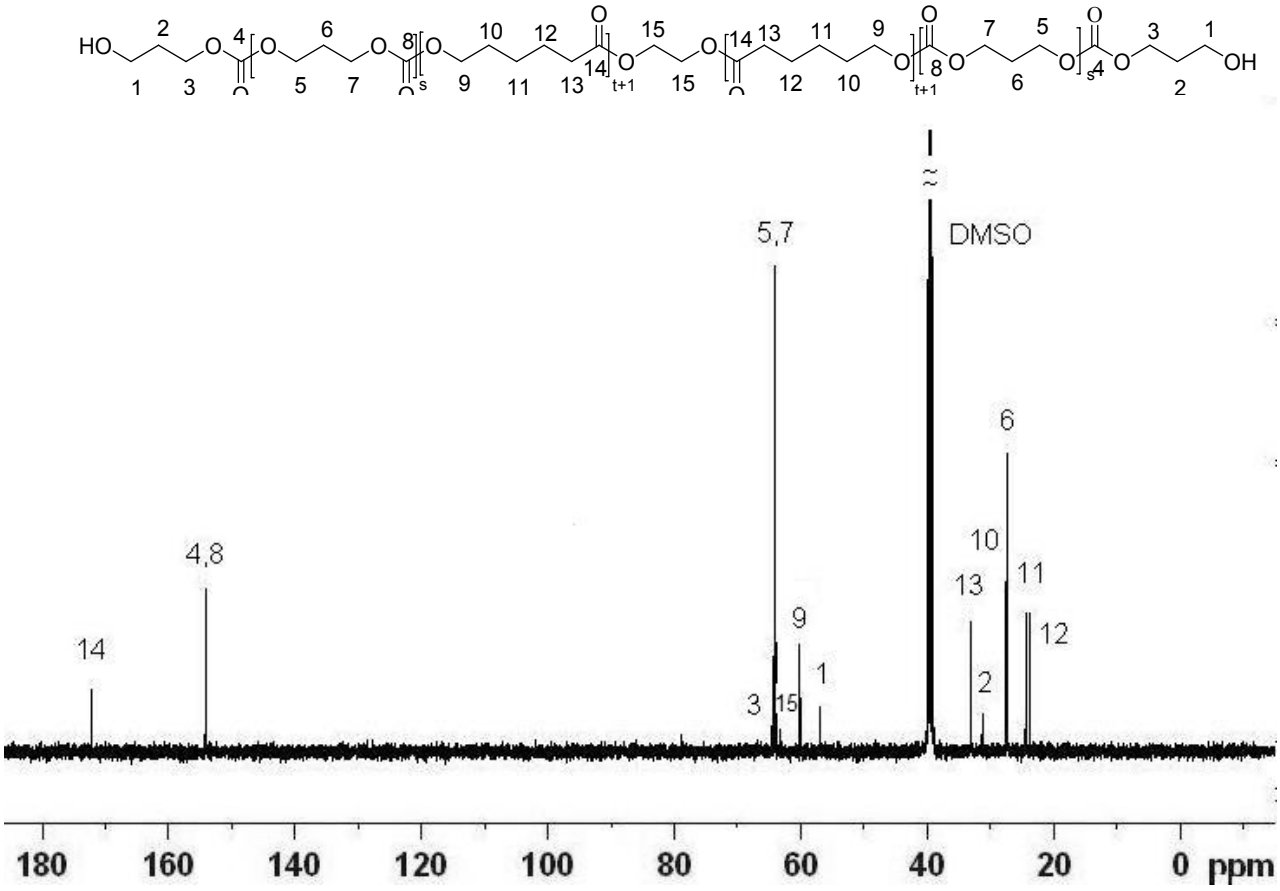


Figure 4.6. DSC spectra of (i) (1) PHB-diol; (2) poly[HB(46 wt%)-*co*-TMC(54 wt%)] (Sample A, Table 4.1); (3) poly[HB(41 wt%)-*co*-TMC(59 wt%)] (Sample C, Table 4.1); (4) poly[HB(32 wt%)-*co*-TMC(68 wt%)] (Sample D, Table 4.1); (5) poly[HB(26 wt%)-*co*-TMC(74 wt%)] (Sample B, Table 4.1); (6) PTMC. (ii) (1) PU_{HBTMC} containing poly[HB(46 wt%)-*co*-TMC(54 wt%)] blocks (sample H, Table 4.3); (2) PU_{HBTMC} containing poly[HB(26 wt%)-*co*-TMC(74 wt%)] blocks (sample I, Table 4.3); (3) PU_{HBCL} containing poly[HB(28 wt%)-*co*-CL(72 wt%)] blocks (sample J in Table 4.3).

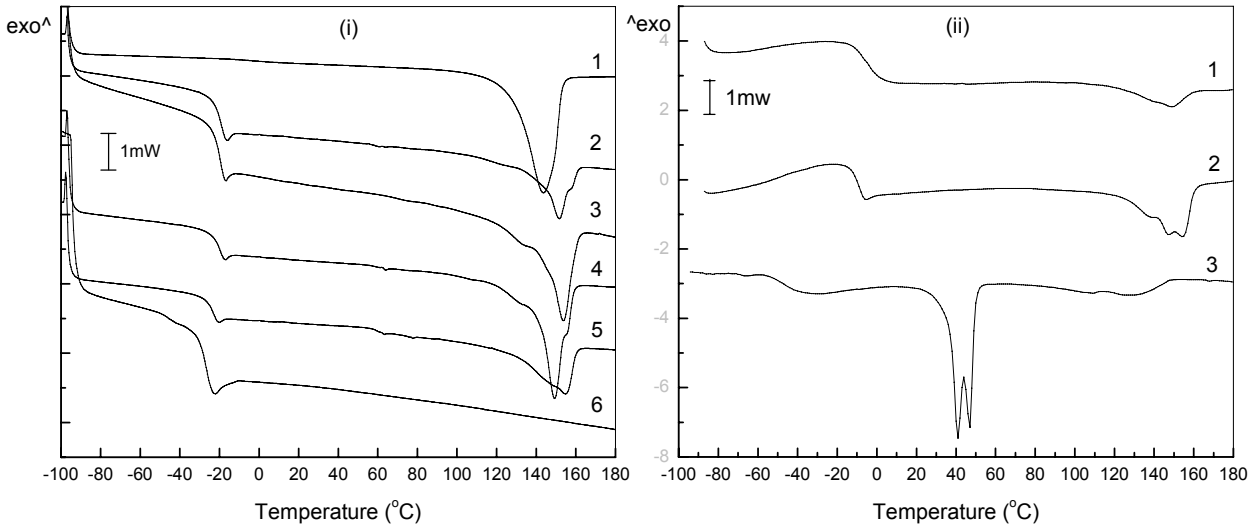


Table 4.2. Physical properties of poly(HB-*co*-TMC)s and poly(TMC-*co*-CL-*co*-TMC) compared with PHB-diol, PCL-diol, and PTMC.

Polymer	Sample Code	M_n (GPC) (g/mol)	M_n (NMR) (g/mol)	CL/TMC [a] (wt%)	HB/TMC [a] (wt%)	T_m [b] (^oC)	T_g [b] (^oC)
PHB-diol		3000	2200			143	-5
PCL-diol[c]		4200	2500			46	-58
PTMC[d]		3400	2000				-27
Poly(HB-*co*-TMC)	A	4500	4800		46 / 54	151	-20
Poly(HB-*co*-TMC)	B	8700	8200		26 / 74	154	-24
Poly(HB-*co*-TMC)	C	5400	5400		41 / 59	153	-21
Poly(HB-*co*-TMC)	D	7100	6900		32 / 68	149	-21
Poly(TMC-*co*-CL-*co*-TMC)	E	10600	10500	29 / 71			-42
Poly(TMC-*co*-CL-*co*-TMC)	F	7700	7700	40 /60			-48
Poly(TMC-*co*-CL-*co*-TMC)[e]	G	7600	7600	38 / 62			-48

a) Calculated based on NMR analysis; b) Obtained from DSC; c) Prepared by enzyme-catalyzed ROP of ε-caprolactone with ethylene glycol as initiator; d) Prepared by enzyme-catalyzed ROP of TMC with water as initiator; e) Scale-up experiment of F.

Preparation of PUs by polymerization of different macro-diols with MDI.

The enzymatically prepared poly(HB-*co*-TMC)s and poly(HB-*co*-CL)[82] have two terminal hydroxyl group and relatively low molecular weight, thus being useful segments for the preparation of block copolymers with high molecular weight and desired thermoplastic properties. Such possibility was explored by the preparation of polyurethanes (PUs) by the polycondensation of MDI with the poly(ester-carbonate) or polyester macro-diols (Scheme 4.2). The polymerizations were performed in DMF at 85 ^{o}C for 12 h without using any catalyst. After reaction, the mixture was treated with methanol and precipitated twice in DMF/methanol (1:4) at 4^{o}C. The polymers were collected by filtration and then dried in vacuum oven at 50 ^{o}C for 24 h. The PUs were obtained in 88%-94% yield with M_n of 34600-53800 g/mol (GPC). The polymerizations and results are summarized in Table 4.3.

Similar to *di*-block poly(HB-*co*-TMC)s and poly(HB-*co*-CL), enzymatically prepared *tri*-block poly(TMC-*co*-CL-*co*-TMC)s is also a reactive micro-diol suitable for the preparation of high molecular weight polymers. Poly(TMC-*co*-CL-*co*-TMC) was thus polycondensed with MDI to prepare PUs. Since it does not contain hard block, poly(TMC-*co*-CL-*co*-TMC) was also polymerized with MDI and PHB-diol to prepare thermoplastic PUs (Scheme 4.2.c-d). Once again, the polymerizations were carried out in DMF at 85 ^{o}C for 12 h without using any catalyst. After similar work-up, the PUs were obtained in 88%-91% yield with M_n of 41500-57200 g/mol (GPC), as summarized in Table 4.3.

Scheme 4.2

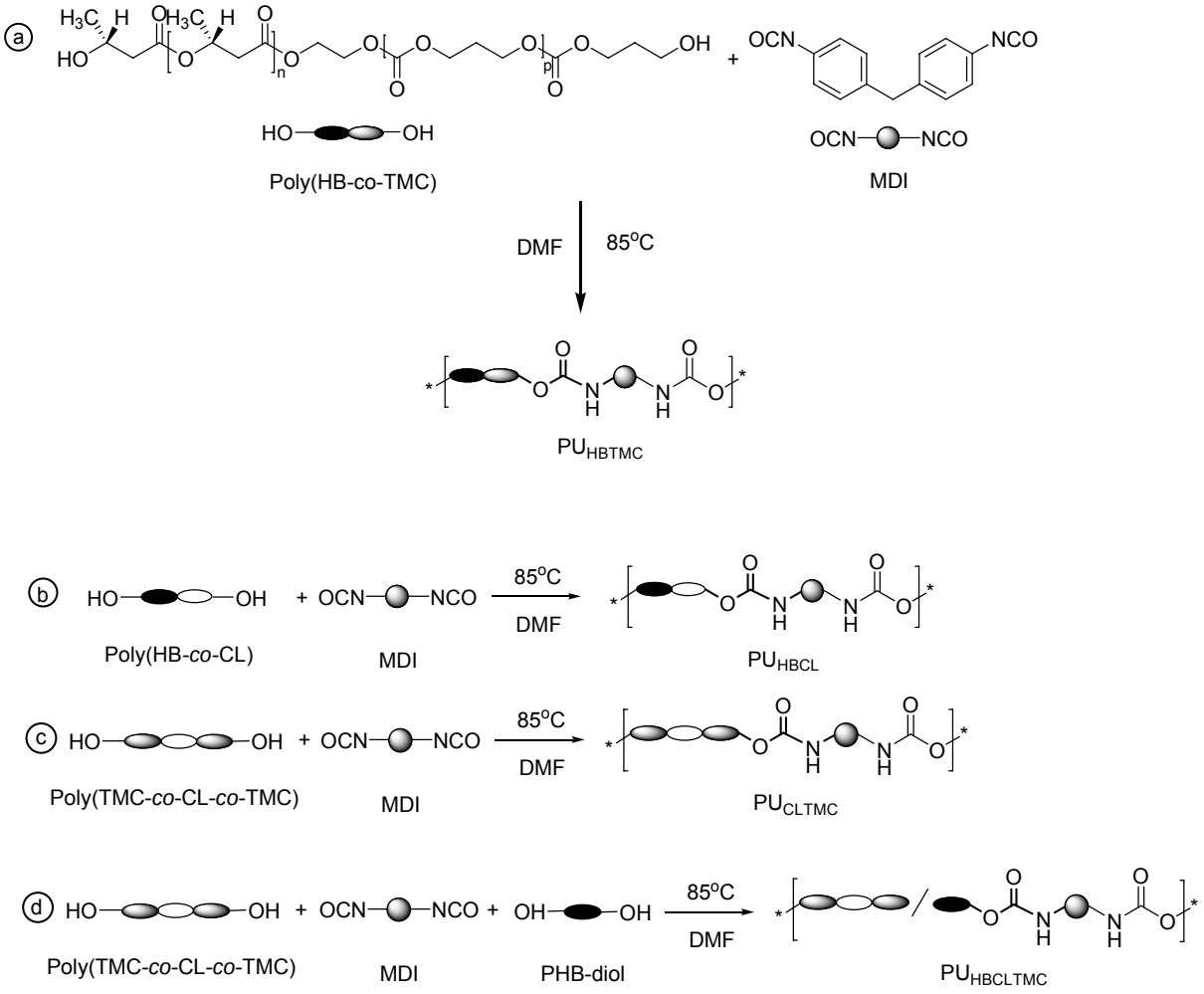

a
Poly(HB-co-TMC)
MDI
DMF
85°C
PU$_{HBTMC}$
b
Poly(HB-co-CL)
MDI
85°C
DMF
PU$_{HBCL}$
c
Poly(TMC-co-CL-co-TMC)
MDI
85°C
DMF
PU$_{CLTMC}$
d
Poly(TMC-co-CL-co-TMC)
MDI
PHB-diol
85°C
DMF
PU$_{HBCLTMC}$

Table 4.3. Synthesis of polyurethanes by polymerization of MDI with poly(HB-*co*-TMC)s, poly(HB-*co*-CL), poly(TMC-*co*-CL-*co*-TMC), and PHB-diol, respectively, in DMF.

Entry	Block-*Co*-polymer-diol	Sample Code	PHB-diol	Diols:MDI (mol:mol)	Temp. (°C)	Time (h)	PU product	Sample Code	M_n (GPC)[a] (g/mol)	Yield (%)
1	Poly(HB-*co*-TMC)	A	-	1:1	85	12	PU_{HBTMC}	H	41800	88
2	Poly(HB-*co*-TMC)	B	-	1:1	85	12	PU_{HBTMC}	I	53800	94
3	Poly(HB-*co*-CL)	X[b]	-	1:1	85	12	PU_{HBCL}	J	34600	89
4	Poly(TMC-*co*-CL-*co*-TMC)	G	-	1:1	85	12	PU_{CLTMC}	K	41500	91
5	Poly(TMC-*co*-CL-*co*-TMC)	G	PHB-diol[c]	1:1	85	12	$PU_{HBCLTMC}$	L	52600	89
6	Poly(TMC-*co*-CL-*co*-TMC)	G	PHB-diol[d]	1:1	85	12	$PU_{HBCLTMC}$	M	57200	88

a) Determined by GPC in DMF at 35 °C with polystyrene as standards; b) Sample from Ref.43; c) Poly(TMC-*co*-CL-*co*-TMC) and PHB-diol were used at a ratio of 50:50 (wt%); d) Poly(TMC-*co*-CL-*co*-TMC) and PHB-diol were used at a ratio of 89:11 (wt %).

Physical and mechanical properties of PUs containing PHB hard block and PTMC or PCL soft block.

The thermal and mechanical properties of PUs (samples H-M) were measured by DSC and UTM, respectively, and the results are summarized in Table 4.4. PU_{HBTMC} (sample H and I) had a T_m of 140-144^oC and T_g of -5 to -9 ^oC, which are lower than those of the corresponding starting materials poly(ester-carbonates) poly(HB-*co*-TMC)s (sample A and B) (Figure 4.6).

PU_{HBTMC} with different weight percentages of PHB and PTMC showed significantly different mechanical properties. PU_{HBTMC} (sample H) containing 46 wt% PHB and 54 wt% PTMC gave rather poor plastic properties, with ε_b of 4.76%, σ_{max} of 6.62 MPa, and E of 285 MPa. On the other hand, PU_{HBTMC} (sample I) containing 24 wt% PHB and 76 wt% PTMC showed excellent plastic properties with ε_b of 252%, σ_{max} of 6.37 MPa, and E of 23 MPa (Figure 4.7). Obviously, the incorporation of more PTMC soft block significantly increased the plastic properties of the PHB-based block copolymers. Thus, we have established a novel method to achieve good mechanical properties for PHB-based block copolymers. The method developed here offers a new way to improve the thermoplastic properties of PHB-based block copolymers by incorporating PTMC blocks at desired weight percentages of PHB and PTMC. The mechanical data for PU_{HBTMC} (sample I) are close to that for several soft tissues.[262] Therefore, such a biodegradable, biocompatible, and thermoplastic block copolymer is potentially useful biomaterial for soft tissue engineering.

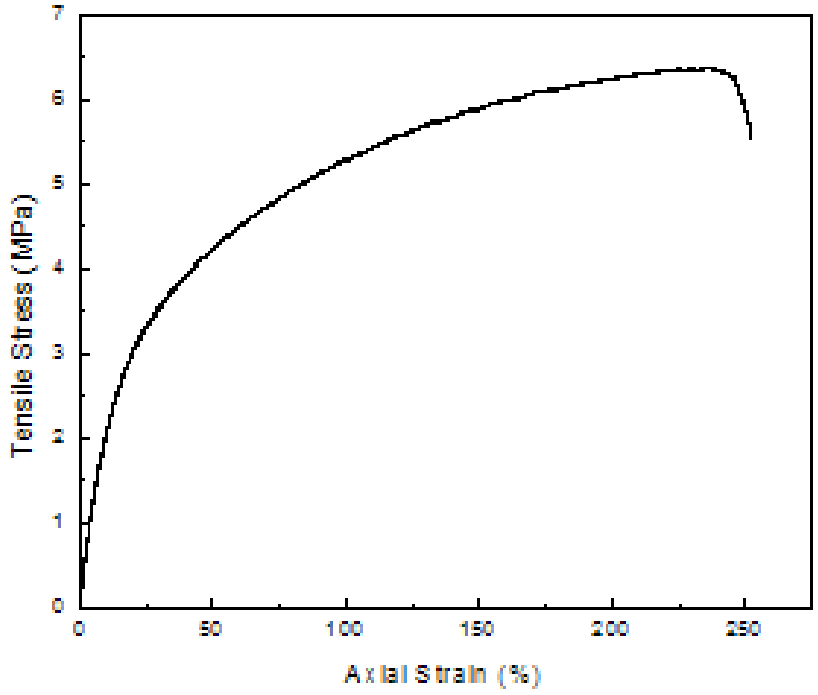
In comparison with PU_{HBTMC} (sample I), the plastic properties of PU_{HBCL} (sample J) containing 28wt% PHB and 72%wt PCL was found to be poor: ε_b of 1.25%, σ_{max} of

Table 4.4. Physical and mechanical properties of PUs containing poly(HB-*co*-TMC), poly(HB-*co*-CL), or poly(HB-*co*-CL-*co*-TMC) blocks.

Entry	PU	Sample code	M_n(GPC) (g/mol)	HB:CL:TMC[a] (wt/wt/wt)	T_m (°C)	T_g (°C)	E[b] (MPa)	σ_y[c] (MPa)	ε_y[d] (%)	σ_b[e] (MPa)	ε_b[f] (%)	σ_{max}[g] (MPa)
1	PU_{HBTMC}	H	41800	46 : 0 : 54	149	-5	285	3.31	1.44	6.61	4.76	6.62
2	PU_{HBTMC}	I	53800	24 : 0 : 76	144	-9	23	1.17	4.84	5.59	252	6.37
3	PU_{HBCL}	J	34600	28 : 72 : 0	46/143	-48	838	2.19	0.40	6.13	1.25	6.25
4	PU_{CLTMC}	K	41500	0 : 38 : 62	ND[h]	ND	19	1.23	5.49	2.64	62.4	4.27
5	$PU_{HBCLTMC}$	L	52600	50 : 19 : 31	ND	ND	155	2.17	1.56	7.04	12.9	7.11
6	$PU_{HBCLTMC}$	M	57200	11 : 34 : 55	ND	ND	105	1.92	1.98	6.45	37.8	7.23

a) Measured by NMR; b) *E*: Young's Modulus; c) σ_y: tensile stress at yield; d) ε_y: elongation at yield (offset 0.1%); e) ε_b: tensile stress at break; f) ε_b : elongation at break; g) σ_{max}: maximum tensile stress; h) ND: Not Detected.

Figure 4.7. Stress–strain curves of PU_{HBTMC} containing poly[HB(26 wt%)-*co*-TMC(74 wt%)] (sample I, Table 4.3).



6.25 MPa, and *E* of 838 MPa. The percentage of PCL soft block in PU_{HBCL} is similar to that of PTMC soft block in PU_{HBTMC} (sample I), thus PTMC is a much better soft block than PCL for adjusting the plastic properties of block co-polymers.

The mechanical properties of PU_{CLTMC} and $PU_{HBCLTMC}$ (sample K, L and M) depend on the PHB component as well as the weight ratio of PCL/PTMC. Without any PHB component, PU_{CLTMC} (sample K) with a ratio of PCL/PTMC of 36/62 (wt/wt) gave ε_b of 62.4%, σ_{max} of 4.27 MPa, and *E* of 19 MPa, being a relatively soft material. Nevertheless, higher percentage of PTMC might be needed to further increase the plastic properties of PU_{CLTMC}. At the fixed weight ratio of PCL/PTMC (36/62), increase of PHB component led to the increase of *E* but also the decrease of ε_b and σ_{max}: $PU_{HBCLTMC}$ containing 11 wt% of PHB (sample M) showed ε_b of 37.8%, σ_{max} of 7.23 MPa, and *E* of 105 MPa. As expected, further increase of PHB component to 50 wt% significantly decreased the plastic properties of $U_{HBCLTMC}$ (sample L): ε_b of 12.9%, σ_{max} of 7.11 MPa, and *E* of 155 MPa.

4.4 Conclusion

Novel block *co*-poly(ester-carbonate)s containing PHB hard block and PTMC soft block with controlled weight ratio of PHB and PTMC as well as controlled molecular weight were synthesized for the first time by enzymatic ring opening polymerization of TMC with PHB-diol. The syntheses are high-yielding, simple, green, and novel. The *di*-block structures of poly(HB-*co*-TMC)s were confirmed by NMR analyses. Poly(HB-*co*-TMC)s with two terminal hydroxyl groups were proven to be useful starting materials for

the further preparation of thermoplastic block co-polymers. Polycondensation of Poly(HB-*co*-TMC)s with MDI afforded block co-polyurethanes (PUs) in high yield, with a T_m of 140-144^oC and a T_g of -5 to -9 ^oC. The mechanical and plastic properties of the PUs were tunable by adjusting the percentage of PTMC components: while PU prepared with poly(46 wt% HB-*co*-54 wt% TMC)s showed rather poor plastic properties, PU prepared with poly(24 wt% HB-*co*-76 wt% TMC)s demonstrated excellent plastic properties with ε_b of 252%, σ_{max} of 6.37 MPa, and E of 23 MPa. The excellent thermal and plastic properties of the latter PU make the biodegradable and biocompatible polymer potentially useful for soft tissue engineering. In comparison, the plastic properties of PU prepared from poly(28wt% HB-*co*-72%wt PCL) was very poor, thus PTMC is a much better soft block than PCL for adjusting the plastic properties of PHB-based block co-polymers.

Block *co*-poly(ester-carbonate)s containing PCL and PTMC soft block with controlled weight percentage of PCL and PTMC as well as controlled molecular weight were also prepared in good yields by enzymatic ring opening polymerization of TMC with PCL-diol. NMR analysis confirmed the expected *tri*-block structure of poly(TMC-*co*-CL-*co*-TMC) containing two reactive hydroxy ending groups. Poly(TMC-*co*-CL-*co*-TMC) was also proven to be suitable starting material for the preparation of PUs with or without the use of PHB-diol as hard segment. PU with a weight ratio of PCL/PTMC of 36/62 (wt/wt) and no PHB gave ε_b of 62.4%, σ_{max} of 4.27 MPa, and E of 19 MPa, being a relatively soft material. PU with 11 wt% of PHB and the same PCL/PTMC ratio showed ε_b of 37.8%, σ_{max} of 7.23 MPa, and E of 105 MPa. Desired properties for special applications might be achieved by adjusting the ratio of PHB, PCL, and PTMC.

CHAPTER 5

ENZYME-CATALYZED POLYCONDENSATION OF POLYESTER MACRO-DIOLS WITH DIVINYL ADIPATE: A GREEN METHOD FOR THE PREPARATION OF THERMOPLASTIC BLOCK COPOLYESTERS

5.1 Introduction

Preparation of block *co*-polymers containing hard and soft segments represents an efficient way for engineering thermoplastic materials. The use of enzyme for such a preparation is of big importance due to the *non*-toxicity and high selectivity of the enzymatic polymerizations. Great successes have been achieved in several types of enzymatic polymerizations such as lipase-catalyzed ring-opening polymerization and polycondensation.[104, 143, 237] While these reactions are now well established for the preparation of homopolymers and random *co*-polymers,[124, 267-270] approaches for the enzymatic preparation of block *co*-polymers are rather rare. Lipase-catalyzed ring-opening polymerization of a lactone with a polymer contacting one hydroxyl ending group such as mono-protected poly(ethylene glycol) (PEG)[271] or two hydroxyl ending group such as PEG[124] or a polyester macro-diol[236] afforded the corresponding block *co*-polymers. This method is useful, but its wide application may be limited due to the fact that only a few lactones can be enzymatically polymerized. On the other hand, lipase-catalyzed polycondensation has a much broader substrate range, and many dicarboxylic acids or their derivatives, glycols, and oxyacids or their esters were successfully used for such polymerization.[176-177, 237-243] Thus, method based on lipase-catalyzed polycondensation for the synthesis of block *co*-polymers could be more general, but it has not been reported so far. Here, we want to develop such a method for the engineering of thermoplastic block *co*-polymer by using two macro-diols as starting materials: one as hard block and another as soft block.

The preparation of block *co*-polyester derived from microbial poly[(*R*)-3-hydroxyalkanoates] (PHAs) was selected as a target. Poly[(*R*)-3-hydroxybutrate]

(PHB) and poly[(*R*)-3-hydroxyoctanoate] (PHO) are the most prominent PHAs and could be produced in large quantities. They are excellent candidates for some biomedical applications due to good biodegradability and biocompatibility.[1-2, 232-233, 244] However, they can not be directly used as thermoplastic biomaterials, since PHB is hard brittle[1, 2] and PHO is soft sticky.[85] Nevertheless, both PHB and PHO can be easily transformed to telechelic PHB-diol and PHO-diol, as the hard and soft segment, respectively, for further polymerization to improve the physical and mechanical properties.[82-83, 87, 245, 272] Block *co*-polymers containing PHB block and one of the other blocks such as poly(ε-caprolactone) (PCL),[83, 245] poly(lactide acid),[245] PEG[87] or PHO[272] were prepared by metal-catalyzed polycondensation using the corresponding macro-diols. However, the use of toxic metal catalysts is not desirable for the preparation of biomedical polymers. Here, we report a novel and green approach for the preparation of block *co*-polymers based on enzyme-catalyzed polycondensation using two polyester macro-diols as substrates and the first enzymatic synthesis of thermoplastic block *co*-polyesters containing PHB and PHO blocks.

5.2 Experimental Section

Materials. Novozym 435 (immobilized *Candida antarctica* lipase B, 10000 PLU/g) was purchased from Novozymes and stored at 4 ^{o}C. Divinyl adipate (99.5%) was purchased from TCI, Japan. Toluene (99.8%) was purchased from Aldrich, chloroform (HPLC, 99.9%) and methanol (HPLC, 99.9%) were obtained from TEDIA. Telechelic

hydroxylated poly-[(*R*)-3-hydroxybutrate] (PHB-diol, M_n of 2170 g/mol, ^{1}H NMR; 3100 g/mol, GPC) and poly-[(*R*)-3-hydroxyoctanoate] (PHO-diol, M_n of 1400 g/mol, ^{1}H NMR; 3200 g/mol, GPC) were prepared according to the published procedures.[82, 272] Novozym 435, PHB-diol, and PHO-diol were dried in vacuum oven at 40°C for 12 hours before use, toluene was dried by refluxing over sodium/benzophenone under argon.

Synthesis of block *co*-polyester poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyoctanoate] by one-step enzymatic polycondensation.

Novozym 435 (40 mg), PHB-diol (98 mg, 0.0452 mmol), and PHO-diol (62.3 mg, 0.0445 mmol) were added in a dry schlenk containing a magnetic stirring bar and then dried under vacuum for 1 h. Under argon atmosphere, divinyl adipate (17.7 μL, 0.0894 mmol) and freshly distilled toluene (2 mL) were added into the schlenk using a dry syringe, followed by stirring at 70 ^{o}C for 8 h. The reaction was terminated by the addition of 10 mL chloroform followed by the removal of Novozym 435 by filtration through filter paper (retention characteristics of 6 μm). Toluene and chloroform were removed under reduced pressure with a rotary evaporator. The raw product was dissolved in 2 mL chloroform, treated with 18 mL methanol, and then precipitated at 4 °C for 8 h. After filtration, the precipitates were dried under vacuum at first with a rotary evaporator and then in a vacuum oven at 40 °C for 24 h. 87.8 mg block poly(HB-*co*-HO) was isolated in 55% yield with a M_n of 9800 g/mol (GPC).

Synthesis of block *co*-polyester poly[(*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyoctanoate] by two-step enzymatic polycondensation

a) Enzymatic condensation of PHB-diol with divinyl adipate. Novozym 435 (100 mg) and PHB-diol (998 mg, 0.446 mmol) were added in a dry schlenk containing a magnetic stirring bar and then dried under vacuum for 1 h. Under argon atmosphere, the freshly distilled toluene (5 mL) and divinyl adipate (884 mg, 4.46 mmol) were added into the schlenk by using a dry syringe. The mixtures were stirred under argon atmosphere at 70 °C for 8 h. The reaction was terminated by adding 25 mL chloroform. Novozym 435 was separated by filtration, followed by removing solvent using a rotary evaporator. The crude product was dissolved in chloroform (2 mL) and precipitated by adding methanol (18 mL) at 4 ^{o}C for 8 h to remove unreacted divinyl adipate. 862 mg PHB-vinyl ester was isolated in 73% yield with a M_n of 2700 g/mol (GPC).

b) Enzymatic polycondensation of PHO-diols with PHB-vinyl ester. PHB-vinyl ester (M_n of 2650 g/mol, ^{1}HNMR; 99 mg, 0.037 mmol), PHO-diol (74 mg, 0.053 mmol), and Novozym 435 (43 mg) were stirred in toluene (2 mL) under anhydrous conditions at 70°C for 8 h. The reaction was terminated by adding 10 mL chloroform. Novozym 435 was separated by filtration, followed by removing solvent using a rotary evaporator. The crude product was dissolved in chloroform (2 mL) and precipitated by adding methanol (18 mL) at 4 ^{o}C for 8 h. 102 mg block poly(HB-*co*-HO) was isolated with a M_n of 14200 g/mol (GPC) in 59% yield.

Measurements. *Gel permeation chromatography (GPC)*. Molecular weight analysis (M_n and polydispersity index M_w/M_n) was performed by using a Waters instrument, with Waters 510 pump, Waters 410 refractive index detector, and Waters HR4E, HR5E and HR6 columns placed in series. THF was used as the eluent for PHB-diol, PHO-diol, and poly(HB-*co*-HO)s measurement at a flow rate of 1.0 mL/min and at 30°C. Sample concentration was about 0.1% (w/v) and the injection volume was 100 μL. Polystyrene standards with molecular weights of 1310, 2970, 13900, 30200, 197000, and 696000 g/mol were used to generate a calibration curve.

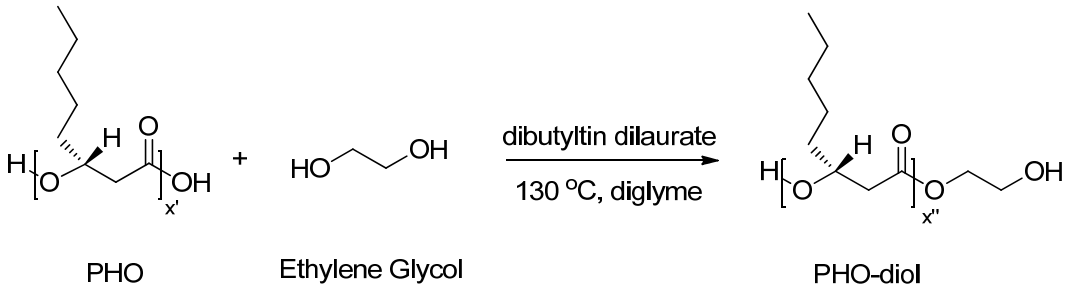
Nuclear magnetic resonance (NMR). ^{1}H-NMR (500 MHz) spectra were recorded with a Bruker AMX500 NMR instrument in DMSO-d_6 at 333K or $CDCl_3$ at room temperature. Chemical shifts were referred to TMS at 0 ppm.

Differential scanning calorimetry (DSC). The thermal properties of polymers were measured on a Mettler Toledo DSC 822 system. Nitrogen was used as purge gas with a flow rate of 20 mL/min. Samples of 10 mg were prepared in aluminum foils, where the aluminum weights of the sample and reference were closely matched. The samples were heated from room temperature to 180 °C with a rate of 20 °C/min, cooled down to -100 °C with a rate of -20 °C/min, and heated again from -100 °C to 180 °C at a rate of 20°C/min. T_m and T_g of the samples were obtained from the second heating curves.

5.3 Results and Discussions

Preparation of PHO-diol

PHO-diol was prepared by the silimar method for PHB-diol synthesis. Transesterification of microbial PHO and ethylene glycol was performed in diglyme at 130 ^{o}C for 4 h (Scheme 5.1). A molecular weight of 2000 was designed for Novozym 435-catalyzed polycondensation with PHB-diol and divinyl adipate. The molecular weight was controlled by the use of catalyst amount. The reaction conditions PHO-diol preparation and the results were summarized in Table 5.1.



Scheme 5.1 Preparation of PHO-diol via transesterification of PHO and ethylene glycol

One-step enzymatic polycondensation for the preparation of block co-polyesters containing randomly arranged PHB and PHO blocks.

PHB-diol with M_n of 3100 g/mol (GPC) and PHO-diol with M_n of 3200 g/mol (GPC) were prepared according to the previously reported procedure.[82, 236, 272] To avoid the

Table 5.1 Preparation of PHO-diol by transesterification of PHO with ethylene glycol in diglyme

Code	PHO	EG	Cat.	Diglyme	Temp.	Time	M_n (GPC)	M_w (GPC)	M_w / M_n	T_g
	g	ml	mg	ml	°C	h	g/mol	g/mol		°C
O1	0.4	0.8	1.5	1.2	120	4	40300	62600	155	
O2	0.4	0.8	3.0	1.2	120	4	29700	49500	1.67	-39
O3	0.4	0.8	6.0	1.2	120	4	22700	35300	1.56	
O4	0.4	0.8	12.0	1.2	120	3	8500	21000	2.47	
O5	0.4	0.8	12.0	1.2	130	4	4400	8400	1.91	-44
O6	0.4	0.8	25	1.2	130	4	3400	6900	2.01	
O7	0.4	0.8	34	1.2	130	4	2000	3800	1.90	
O8	4.0	8.0	250	12	130	4	3200	6000	1.88	-44

EG: Ethylene Glycol, Cat. : Dibutyltin dilaurate

possible transesterification between the polyester-diols, enzymatic polycondensation was carried out by using the two polyester macro-diols with an active divinyl ester. One-step polycondensation of PHB-diol, PHO-diol, and divinyl adipate with Novozym 435 as the catalyst was first investigated (Scheme 5.2). PHB-diol and PHO-diol have similar structures and thus similar chances to react with vinyl adipate, giving rise to the formation of poly(HB-*co*-HO)s with randomly arranged PHB and PHO blocks. The reactions were carried out in dry toluene at 70 ^{o}C under argon atmosphere at a molar ratio of PHB-diol, PHO-diol, and vinyl adipate of 1:1:2 (Table 5.2, entry 1). The generated acetaldehyde was easily released at this temperature to drive the reaction to the formation of polymer. After 8 h reaction, the solvent in the reaction mixture was removed by evaporation under vacuum, the residue was dissolved in chloroform (2 mL), treated with methanol (18 mL), and precipitated at 4 ^{o}C for 8 h. The product was dried in a vacuum oven at 40 ^{o}C for 24 h to give a yield of 55%. The molecular weight (M_n) of the polymer (Sample A, Table 5.2) was determined by GPC as 9800 g/mol, which is much higher than those of the starting materials PHB-diol and PHO-diol (Figure 5.1). This confirmed also the occurrence of enzymatic polycondensations.

^{1}H NMR spectra of PHO-diol, PHB-diol, and polymer sample A were given in Figure 5.2 (i-iii). The number of HO repeating unit x of PHO-diol was established as 9.3 based on the signal ratio of protons *b* and *f* in Figure 5.2 (i), while the number of HB repeating unit (n-1) in PHB-diol was deduced as 23.6 from the signal ratio of protons *m* and *u* in Figure 5.2 (ii). In Figure 5.2(iii), signals of protons *t'* and *tt* from the backbone of the junction unit were clearly observed, while signals of the protons of the vinyl ester

Scheme 5.2 Preparation of block co-polyesters by enzyme-catalyzed polycondensation of PHB-diol, PHO-diol, and divinyl adipate *via* one- or two-step reactions.

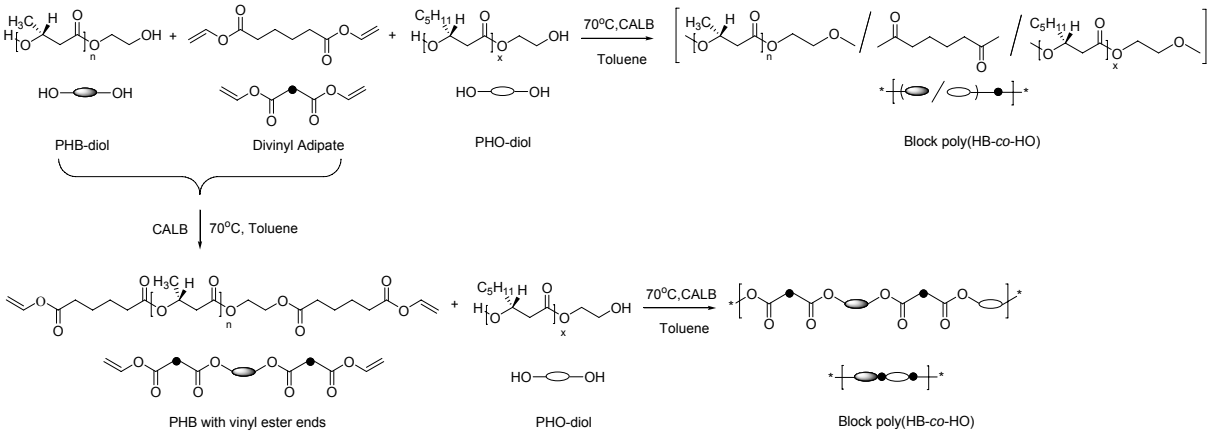


Table 5.2. Preparation of block co-polyester poly(HB-co-HO) by Novozym 435-catalyzed polycondensation of PHB-diol, PHO-diol, and divinyl adipate *via* one-step or two-step reactions.

Entry	Oligomer (1)	M_n [a] g/mol	Oligomer (2)	M_n [b] g/mol	DA [c] (3)	Feed Ratio (1):(2):(3)[d]	Feed Ratio (1+2):E[e]	M_n [f] g/mol	M_w [g] g/mol	M_w/M_n	Yield %	HB / HO[h]	T_m (°C)	T_g (°C)	Code
1	PHB-diol [i]	2170 [j]	PHO-diol	1400 [k]	DA	1:1:2	4:1	9800	16200	1.66	55	1 / 0.68	142/153	-37	A
2	PHB-vinyl ester [l]	2650	PHO-diol	1400	-	1:1:-	4:1	9200	16300	1.76	62	1 / 0.77	139/149	-37	B
3	PHB-vinyl ester [l]	2650	PHO-diol	1400	-	1:1.5:-	4:1	14200	25100	1.77	59	1 / 1.00	136/142	-39	C
4	PHB-vinyl ester [l]	2650	PHO-diol	1400	-	1:2:-	4:1	8800	15800	1.80	55	1/ 1.28			

a,b: Calculated from ^{1}H NMR; c: Divinyl adipate; d: Molar ratio; e: E for enzyme, weight ratio; f,g: From GPC; h: Molar ratio calculated from ^{1}H NMR spectra. i: one-step polycondensation in toluene at 70°C for 8 h; j: 3100 from GPC; k: 3200 from GPC; l: two-step polycondensation: PHB-vinyl ester was prepared by reaction of PHB-diol and divinyl adipate in the presence of Novozyme 435 in toluene at 70°C for 8 h; the second step reaction was also performed in toluene at 70°C for 8 h.

Figure 5.1. GPC spectra of (i) PHB-diol; (ii) PHO-diol; (iii) Block poly(HB-*co*-HO) prepared from one-step polycondensation (Sample A in Table 5.2); (iv) PHB-vinyl ester prepared from the first step reaction in the two-step polycondensation; (v) Block poly(HB-*co*-HO) prepared from two-step polycondensation (Sample C in Table 5.2).

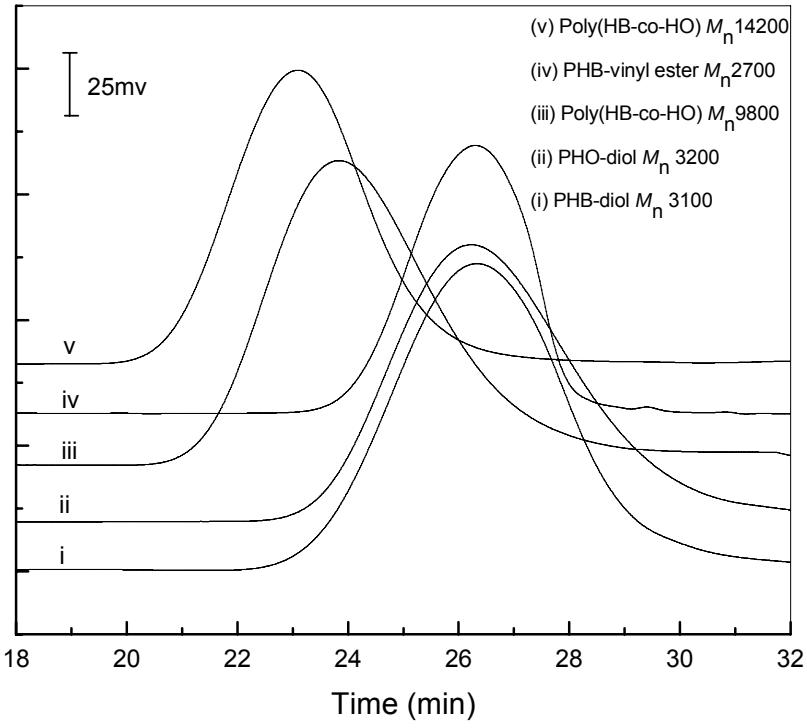


Figure 5.2. ^{1}H NMR spectrum in DMSO-$_{d6}$ at 333K of (i) PHO-diol; (ii) PHB-diol and (iii) Poly(HB-*co*-HO) prepared from one-step polycondensation (Sample A in Table 5.2);

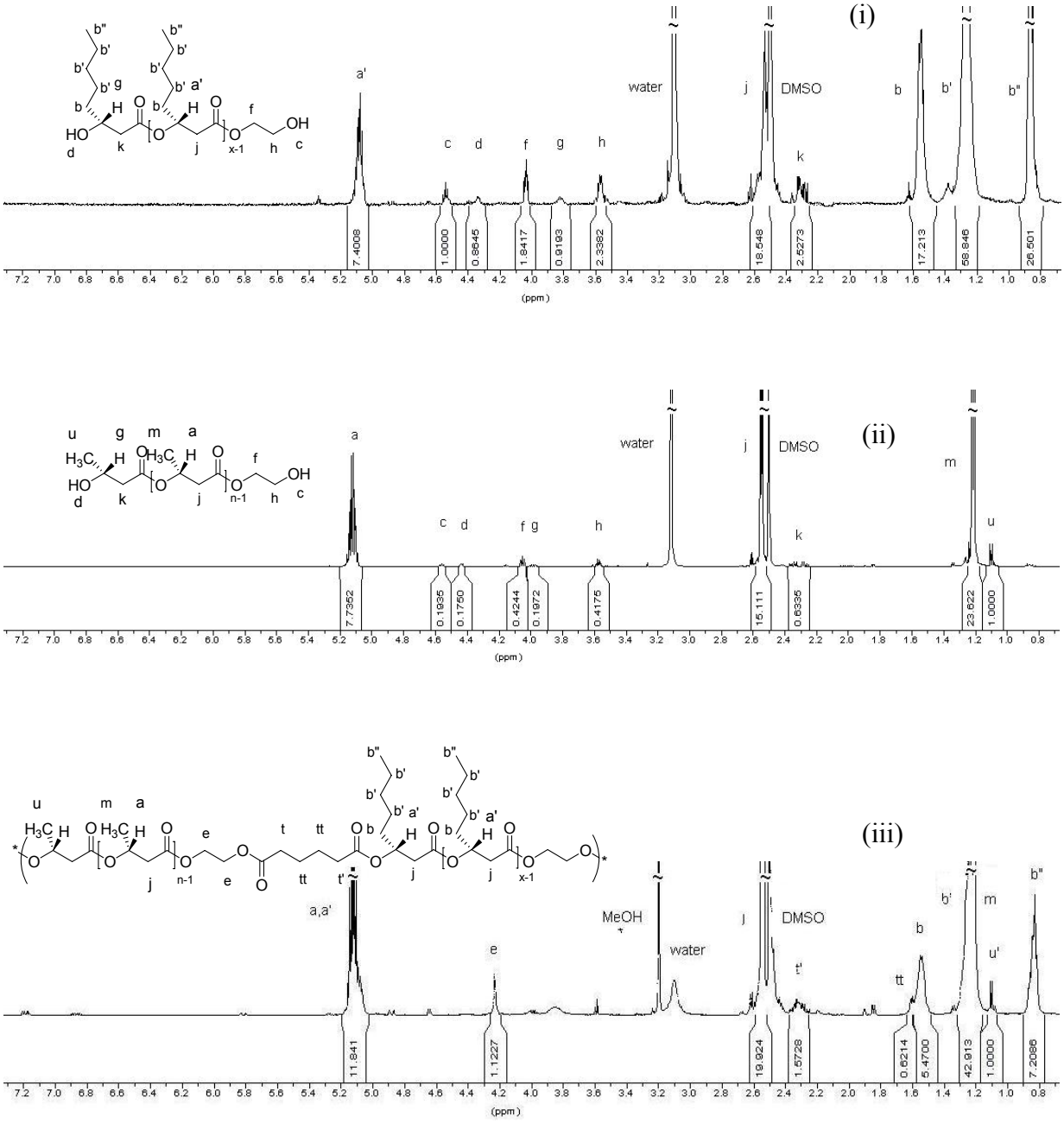
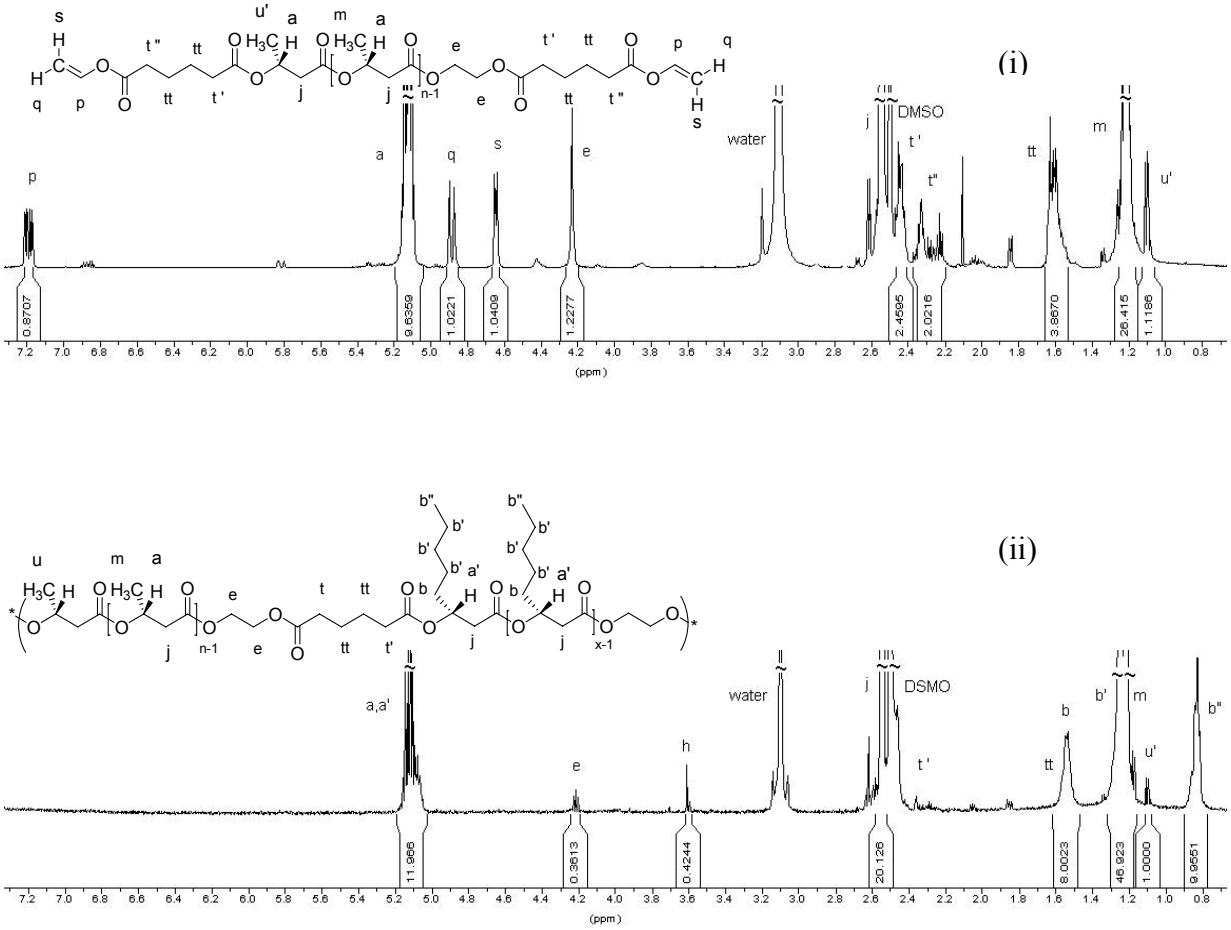


Figure 5.3. ^{1}H NMR spectrum in DMSO-$_{d6}$ at 333K of (i) PHB-vinyl ester prepared from the first step of two-step polycondensation; (ii) Poly(HB-*co*-HO) prepared from two-step polycondensation (Sample C in Table 5.2).



ending group were hardly visible. This indicates that divinyl adipate was reacted and the backbone was incorporated into the polymers. On the other hand, signals of OH groups (protons *c* and *d*) of PHB-diol and PHO-diol disappeared, small amount of the signals of protons *f*, *g*, and *h* remained, and new peak of proton *e* was clearly observed in Figure 5.2 (iii). These data suggest that PHB-diol and PHO-diol were reacted and incorporated into the polymers, and part of the OH ending groups of PHO-diol or PHB-diol severed as the ending groups of the final polymer. The other signals from the backbone of PHB (proton *m*, *a*, and *j*) and PHO (proton *b''*, *b'*, *b*, *a'*, and *j*) were easily assigned, as shown in Figure 5.2(iii). The signal intensity for *b''* (3 H) was 7.2, thus the intensity of *a'* (1 H) should be 2.4; the total signal intensity for *a* and *a'* is 11.8, therefore the intensity of a should be 9.4; considering *n* of 24.6 for PHB block and *x* of 9.3 for PHO block, the ratio of PHB / PHO block can be calculated as (9.4/24.6)/(2.4/9.3) = 1/0.68. Combining with the results on molecular weights determined by GPC, it could be concluded that the final polymer contains on average three randomly arranged PHA blocks with a ratio of PHB and PHO blocks of 1:0.68.

Two-step lipase-catalyzed polycondensation for the preparation of block co-polyesters containing A-B type arranged PHB and PHO blocks.

To control the structure of the block copolymers, two-step lipase-catalyzed polycondensation was conducted (Scheme 5.2). Theoretically, block co-polyester with A-B type arranged blocks should be produced. Reaction of PHB-diol with 10 fold divinyl adipate was performed in the presence of Novozym 435 in dry toluene under argon

atmosphere at 70 °C for 8 h. The unreacted divinyl adipate was effectively washed out with chloroform, and the product was precipitated in chclorofom/methanol (2mL/18 mL) at 4 ^{o}C. This gave the corresponding product PHB-vinyl ester (M_n of 2700 g/mol, GPC) in 73% yield. Reaction of PHB-vinyl ester with PHO-diol in a molar ratio of 1:1-2 was carried out with Novozym 435 in toluene at 70°C for 8 h. The reaction conditions and results were summarized in entry 2-4 of Table 5.2. After same work-up procedure as described above for one-step polycondensation, poly(HB-*co*-HO)s were obtained in 55-62% yield. GPC analysis showed a significant increased molecular weight of the products (M_n of 8800-14200 g/mol) compared with the starting materials (Figure 5.1). The use of 1:1.5 ratio led to higher M_n of the final polymer than the use of either 1:1 ratio or 1:2 ratio.

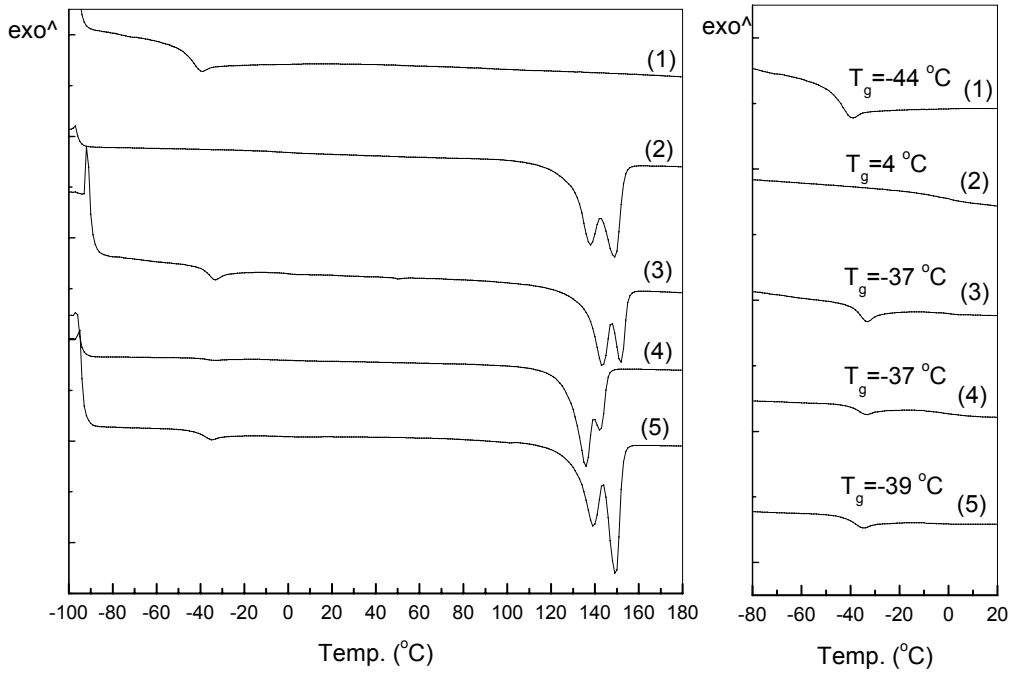
The ^{1}H NMR spectrum of PHB-vinyl ester was showed in Figure 5.3 (i). The signals of OH group (protons *c* and *d*) and the protons *f*, *g*, and *h* of PHB-diol disappeared, while a new peak of proton *e* was observed. This suggested that the OH groups were reacted with divinyl adipate. The signals of protons *t'*, *t"*, and *tt* of the vinyl ester part split into multiple peaks, thus being significantly different from those of divinyl adipate. This suggested the happening of the condensation of divinyl adipate with PHB-diol. Other protons (*p*, *q*, *s*) of vinyl ester part were clearly observed, showed the signal ratio of 1:1:1, and were proportional to the signals of *t'*, *t"*, *tt*. Signals of protons *a*, *j*, and *m* from backbone of PHB remained in the spectrum. Based on the signal intensity of protons *a* and *p*, the ratio of PHB and the vinyl ester part can be established as (9.6/24.6)/0.87 = 1:2.2. Combining with the results from GPC, it could be concluded that the obtained product is PHB containing two vinyl ester ending groups.

The ^{1}H NMR spectrum of poly(HB-*co*-HO) (Sample C, Table 5.2) was shown in Figure 5.3 (ii). The signals of protons *p*, *q*, *s* from the PHB-vinyl ester disappeared totally, and the signals of protons *c*, *d*, *f*, *h*, and *g* from the ending group of PHO diol were either disappeared or largely reduced. This indicated that the OH group from PHO block was mostly reacted with the vinyl ester groups forming block *co*-polyester and partially remained as end group of the final polymer. The protons (*m*, *a*, *j*, *b"*, *b'*, *b*, and *a'*) from the backbone of PHB and PHO absorbed in the expected areas. Similarly, the ratio of PHB/PHO block can be calculated based on proton *a* and *a'*: the signal intensity for *b"* (3 H) was 10, thus the intensity of *a'* (1 H) should be 3.3; the total signal intensity for *a* and *a'* was 12.0, thus the intensity of proton *a* should be 8.7; the ratio of PHB/PHO block can be estimated as (8.7/24.6)/(3.3/9.3) =1:1. As the M_n was determined by GPC as 14200 g/mol, the polymer sample C contains on average about 5 PHA blocks at a ratio of PHB and PHO blocks of 1:1 with A-B-A-B-A type structure. In the ^{13}C NMR spectrum, signal at δ=168.5 and 168.3ppm were observed, which belong to the carbon from carboxyl group of PHB and PHO, respectively. No other signals were found between 168.3 and 168.5ppm, indicating no random polymer of PHB and PHO.

Physical properties of block co-poly(HB-*co*-HO)s

The melting temperature (T_m) and glass transition temperature (T_g) of block poly(HB-*co*-HO)s were analyzed by DSC (Figure 5.4) and summarized in Table 5.2. All block *co*-polymers have T_m of 142-153 ^{o}C and 136-142 ^{o}C, and T_g from -37 ^{o}C to -39 ^{o}C.

Figure 5.4. DSC spectra of (1) PHO-diol; (2) PHB-diol; (3) Poly(HB-*co*-HO) M_n of 9800 (Sample A in Table 5.2); (4) Poly(HB-*co*-HO) M_n of 9200 (Sample B in Table 5.2); (5) Poly(HB-*co*-HO) M_n of 14200 (Sample C in Table 5.2).



The enzymatically prepared block co-poly(HB-*co*-HO) demonstrated good thermal properties, thus being potentially useful thermoplastic materials for biomedical applications.

5.4 Conclusion

A new, green and efficient method for the preparation of block *co*-polyesters *via* Novozym 435-catalyzed polycondensation of polyester macro-diols and divinyl adipate was successfully demonstrated. Thermoplastic *co*-polyesters containing microbial poly[(*R*)-3-hydroxybutyrate] (PHB) and poly[(*R*)-3-hydroxyoctnoate] (PHO) blocks were enzymatically prepared for the first time, by either one- or two-step enzymatic polycondensation of PHB-diol (M_n of 3100 g/mol, GPC), PHO-diol (M_n of 3200 g/mol, GPC) with divinyl adipate. While one-step synthesis is simpler and gave block *co*-polyester poly(HB-co-HO) (M_n of 9800 g/mol, GPC) with randomly arranged blocks, two-step polycondensation is more controllable and afforded block co-polyester poly(HB-*co*-HO) (M_n of 8800-14200 g/mol, GPC) with A-B type arranged blocks. The enzymatically prepared block copolymer poly(HB-*co*-HO)s demonstrated good thermal properties with T_m of 136-142 ^oC and 142-153 ^oC, and T_g of -37 to -39 ^oC, thus being potentially useful thermoplastic biodegradable and biocompatible materials

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Novel polymeric biomaterials with potential biomedical applications were prepared by enzymatic modification of microbial polyester. A novel method of preparing block copolymers was established for the preparation of biomaterials *via* low-molecular-weight telechelic diol-initiated enzymatic ring opening polymerization of cyclic monomers. PHB-diol initiated ring-opening polymerization of ε-caprolactone (CL) was the first example of using a telechelic macro-diol containing ester groups as an initiator for the enzymatic ring opening polymerization. There were no tranesterification occurred during the ring opening polymerization, thus the backbone structure of PHB-diol was well kept and block copolymers of poly(HB-*co*-CL) with well-defined structures were successfully prepared.

Alcohols are the commonly used initiator for ring opening polymerization of cyclic lactones. The OH group provided from different alcohols is the functional group during the initiation step of the ring opening polymerization. PHB-diol has a primary OH and a secondary OH group at each end of its chain structure. Although it has much longer carbon chain than simple alcohols, it is still possible to provide OH group with enough activity for the initiation step of ring opening polymerization of CL.

Novozym 435 showed high selectivity between the primary and the secondary OH from the PHB-diol ending groups and *di*-block copolymers of poly(HB-*co*-CL) were synthesized. The primary OH group from PHB-diol demonstrated higher activity than

that of the secondary OH in the initiation stage of the enzymatic ring opening polymerization. After ring opening stage of the cyclic monomers, the linear monomer unit linked to the enzyme and formed an acyl enzyme complex. Then, the linear monomer unit was released from the acyl enzyme complex and transferred to an active OH group. The primary OH from PHB-diol with higher activity was more competitive to accept the linear monomer unit from the acyl enzyme complex than the secondary OH. Thus, the chain growth only occurred in the primary OH end, and the secondary OH was un-reacted during the polymerization. As a result, *di*-block copolymers were prepared in the ring opening polymerization of CL.

This estabalished method was extened for PHB-diol initiated ring-opening polymerization of cyclic trimethylene carbonate (TMC). Thermoplastic di-block copolymers containing PTMC block as soft domain and PHB block as hard domain were synthesized *via* enzymactic ring-opening polymerization for the first time. To show the selectivity of Novozym 435, PCL-diol and poly(HB-*co*-CL) with different –OH terminal groups were also applied as macro-diol initiator for the ring-opening polymerization of TMC. The corresponding di-block or tri-block copolymers were obtained. Initiator with both primary –OH as functional group at both ends demonstrated a A-B-A type tri-block structure of the copolymers. However, poly(HB-*co*-CL) with one primary –OH and one – secondary –OH as ending groups showed a A-B-C type tri-block structure.

Another novel method for the preparation of block polyester *via* polycondensation of telechelic macro-diols with activated dicarboxylic acid ester was reported for the first

time. Thermoplastic copolyesters containing poly[(*R*)-3-hydroxybutyrate] (PHB) and poly[(*R*)-3-hydroxyoctonoate] (PHO) blocks were enzymatically prepared by one-step or two-step Novozym 435-catalyzed polycondensation of PHB-diol, PHO-diol with divinyl adipate as junction units. The one-step synthesis is simpler and gave copolyester of poly(HB-*co*-HO)s with randomly arranged PHB and PHO blocks, while the two-step polycondensation demonstrated more defined structure of A-B type arranged PHB and PHO blocks.

The established methods provide efficient routes to prepare block copolymers with well-defined backbone structures. The novel and selective enzymatic ring-opening polymerization has been proved to be a new synthetic method for preparing *di*-block or *tri*-block copolymers with functional end groups, which could also be modified for further applications.

Based on the established novel methods, different type of block copolymers were prepared with desired backbone structure and improved thermal and mechanical properties. *Di*-block co-polyesters containing PHB and PCL blocks were synthesized in high yield *via* enzymatic ring-opening polymerization of ε-caprolactone with PHB-diol. The structures of the *di*-block polymers with two different OH end groups were established by IR, ^{1}H-NMR and ^{13}C-NMR analyses. Poly(HB-*co*-CL)s with 44-74%(w/w) PCL demonstrated good thermal properties with T_g of about -60°C and T_m of 120-149°C and 50-54°C. *Di*-block co-polyester-carbonates contained PHB and PTMC blocks were also prepared *via* enzymatic ring-opening polymerization of trimehtylene carbonate or

propylene carbonate with PHB-diol. By incorporating PTMC blocks (54% - 74%) into PHB block, the T_g of poly(HB-*co*-TMC) were modified from -4°C to -24°C. The *tri*-block copolymers of poly(TMC-*co*-CL-*co*-TMC) *via* PCL-diol initiated ring-opening polymerization of TMC showed lower T_g of -42°C and -48 °C with 60% and 71% PTMC block contents, respectively. Poly(HB-*co*-CL), poly(HB-*co*-TMC), and poly(HB-*co*-CL-*co*-TMC) can be further polymerization with MDI to produce the corresponding polyurethanes, which showed T_g of -5 to -48°C.

The improvement of mechanical properties of the prepared block copolymers were mainly studied and compared by the corresponding polyurethans prepared *via* enzymatic synthesized polyesters or poly(ester-carbonate)s with MDI. The mechanical properties can be modified by changing the components combination and the ratio between different polymer components. As PHB and PCL were semi-crystalline polyesters, they contribute greatly on the crystalline ability of the corresponding block copolymers. Thus the content of PHB or PCL has significant effect on the Young's modulus.

Their *di*-block structures of poly(HB-*co*-CL), poly(HB-*co*-TMC) and poly(TMC-*co*-CL-*co*-TMC) helped to modify their physical and mechanical properties by changing the molar ratio of different components content and the polymer chain length, thus being potentially useful thermoplastic biomaterials.

In this thesis, the macrodiol-initiated ring opening polymerization of cycilic lactone or carbonate is a new, efficient and green method in preparation of *di*- or *tri*-block copolymer using enzymatic catalyst without involving any junction units, regular

polyester chains or polyester-carbonate chains were formed, which may make the biodegradation rate be predictable and controlable. The poly(HB-co-HO)s are novel PHA-based block copolymers with both segments are naturally origined, thus has prominent biocompatibility and biodegradability. The block copolymers synthezied in this thesis are competitive candidate in soft tissue engineering, such as degradable scaffolds, flexible stent, drug carrier in controlled delivery.

6.2 Future Work

Due to the limited monomers for the enzymatic ring opening polymerization, only two cyclic monomers of CL and TMC were studied for PHB-based block copolymer synthesis in this thesis, and showed desired potential to be used as biomaterials for clinical applications. Substituted cyclic lactones or carbonates may be another family of potential monomers for the enzymatic ring-opening polymerization to prepare PHB-based block copolymers. As reviewed in chapter 1, the enzymatic ring-opening polymerization of substituted cyclic lactones has been investigated. However, the monomer conversion and the polymerization degree were not so attractive for practical applications. A possible reason is the stero-resistance caused by the substituted group from the cyclic monomer. However, this substituted group may be important as a side chain in the corresponding linear polymers to modify and control the physical and mechanical properties. To overcome this conflict, possible method is figure out by modifying the enzyme configuration to provide various space, thus the catalytic sites from the enzyme can

accept more cyclic monomers with different substituted positions or different chain length of substituted group.

With the succeeded example of telechelic macro-diol as initiator for the ring-opening polymerization of cyclic monomers, multi-functionalized low-molecular-weight polymers may also be applied for the enzymatic ring-opening polymerization of cyclic monomers to produce possible dendrimers. The number of functional groups and the length of each arms may also be controllnable to provide tunable physical and mechanical properties of the new polymers.

Enzymatic ring-opening polymerization was accompanied with the possible hydrolysis as a reversible reaction. To improve the polymerization degree and thus achieve higher monomer conversion with longer chain length, it would be interesting to control the hydrolysis reaction. Water amount is an important factor that can significantly affect the catalytic activity of an enzyme. A favorite amount of water may be needed to bond with the surface to the enzyme, and help to keep the activity. There have been some studies on the water effect on the enzymatic polymerization of CL, however, more systematic work might be necessary for other cyclic monomers due to the different polarity. The behavior of water in the reaction system would also be a potential direction to understand the mechanism of ring-opening polymerization. It is possible that the water bonded to the enzyme be released under specific conditions, the release water molecules may be an active factor for the unfavored hydrolysis. How to control the bonded water and avoid the released free water may be essential to control the side reaction.

Enzymatic polymerization is normally performed in organic media. The log *P* value of different organic solvent have been reported to paly an important role in the

reaction by maintaining the water layer on the surface of the enzyme. A combination of different organic solvent to form a new reaction media may be helpful to provide a wider range of log *P* value.

In this thesis, we reported the enzymatic polycondensation of macro-diols with activated dicarboxylic acid ester for polyester synthesis for the first time. Based on this method, macro-diols with different chain length may be studied to produce block copolymers with different properties. In addition, the functionalization of polymers into macro-diol is a simple and well-studied method, thus it is possible that more biocompatible or biodegradable polymers can be applied in enzymatic polycondensation for biomaterials preparation. On the other hand, this method can also be extended by functionalize a polymer candidate into vinyl-ester ended active starting materials for the enzymatic polymerization with macro-diols.

References

1. Doi, Y., *Microbial Polyester*. VCH: New York, 1990.

2. Mobley, D. P., *Plastics from Microbes*. Hanser: Munich, 1994.

3. Williams, S. F.; Martin, D. P. Therapeutic uses of polymers nd oligomers comprising gamma-hydroxybutyrate. US Patent 6623730, 2000.

4. Williams, S. F.; Martin, D. P.; Skraly, F. A. Medical devices and applications of polyhydroxyalkanoate polymers. US patent 7268205, 2000.

5. Lemoigne, M. *CR Acad Sci* **1925,** 180, 1539-1541.

6. Lemoigne, M. *Ann Inst Pasteur* **1925,** 39, 144.

7. Lemoigne, M. *Bull Soc Chim Biol* **1926,** 8, 770-782.

8. Lemoigne, M. *Ann Inst Pasteur* **1926,** 41, 148-165.

9. Williamson, D. H.; Wilkinson, J. F. *J. Gen. Microbiol* **1958,** 19, 198-209.

10. Anderson, A. J.; Dawes, E. A. *Microbiological Reviews* **1990,** 54, 450.

11. Holmes, P. A., *Development in Crystalline Polymers*. Elsevier: New York, 1988; Vol. 2.

12. Steinbuchel, A., *Biomaterials*. MacMillan: London, 1991.

13. Muller, H. M.; Seebach, D. *Angew Chem. Int. Ed.* **1993,** 32, 477.

14. Holmes, P. A.; Wright, L. F.; Collins, S. H. EP 52495, 1982.

15. M. Avella, E. M. a. M. R. *Journal of Materials Science* **2000,** 35, (3), 523-545.

16. Barham, P. J.; Keller, A.; Otun, E. L.; Holmes, P. A. *J. Mater. Sci.* **1984,** 19, 2781-2794.

17. Barham, P. J. *J. Mater. Sci.* **1984,** 19, 3826-3834.

18. Doi, Y.; Kunioka, M.; Nakamura, Y.; Soga, K. *Macromolecules* **1986,** 19, 1274.

19. Williams, S. F. M., D. P.; Horowitz, D. M.; Peoples, O. P. *Int. J. Biol. Macromol.* **1999,** 25, 111-121.

20. Chen, Q. W., Q. *Biomaterials* **2005,** 26, 6565-6578.

21. Holmes, P. A., *Development in Crystalline Polymers*. Elsevier: London, 1988.

22. Howells, E. R. *Chem. Ind.* **1982,** 8, 508-511.

23. Barham, P. J.; Keller, A. *J. Poly. Sci.: Poly. Phys.* **1986,** 24, 69-77.

24. de Koning, G. J. M.; Lemstra, P. J. *Polymer* **1993,** 34, 4089-4094.

25. De Koning, G. J. M.; Scheeren, A. H. C.; Lemstra, P. J. *Polymer* **1994,** 35, 4598-4605.

26. Hobbs, J. K.; Barham, P. J. *J. of Mater. Sci.* **1998,** 33, 2515-2518.

27. Furuhashi, Y.; Imamura, Y.; Jikihara, Y.; Yamane, H. *Polymer* **2005,** 45, 5703-5712.

28. Mitomo, H.; Doi, Y. *International Journal of Biological Macromolecules* **1999,** 25, 201-205.

29. Strobl, G. R., *The Physics of Polymers Concepts for Understanding Their Structures and Behavior*. Springer: Verlag, 1996.

30. Avella, M.; Martuscelli, E. *Polymer* **1988,** 29, 1731-1737.

31. Greco, P.; Martuscelli, E. *Polymer* **1989,** 30, 1475-1483.

32. Paglia, E. D.; Beltrame, P. L.; Canetti, M.; Seves, A.; Marcandalli, B.; Martuscelli, E. *Polymer* **1993,** 34, 996-1001.

33. Sadocco, P.; Canetti, M.; Seves, A. *Polymer* **1993,** 34, 3368-3375.

34. Scandola, M.; Ceccorulli, G.; Pizzoli, M. *Macromolecules* **1992,** 25, 6441-6446.

35. Siciliano, A.; Seves, A.; De Marco, T.; Cimmino, S.; Martuscelli, E.; Silvestre, C. *Macromolecules* **1995,** 28, 8065-8072.

36. Canetti, M.; Sadocco, P.; Siciliano, A.; Seves, A. *Polymer* **1994,** 35, 2884-2887.

37. Cimmino, S.; Iodice, P.; Martuscelli, E.; Silvestre, C. *Thermochimica Acta* **1998,** 321, 89-98.

38. Xing, P.; Dong, L.; An, Y.; Feng, Z.; Avella, M.; Marcandalli, B. *Macromolecules* **1997**, 2726-2733.

39. Xing, P.; Ai, X.; Dong, L.; Feng, Z. *Macromolecules* **1998,** 31, 6898-6907.

40. Park, J. W.; Doi, Y.; Iwata, T. *Biomacromolecules* **2004,** 5, 1557-1566.

41. Xu, J.; Guo, B.; Zhou, J.; Li, L.; Wu, J.; Kowalczuk, M. *Polymer* **2005,** 46, 9176-9185.

42. Pearce, R.; Jesudason, J.; Orts, W.; Marchessault, R. H. *Polymer* **1992,** 33, 4647-4649.

43. Pearce, R.; Brown, G. R.; Marchessault, R. H. *Polymer* **1994,** 35, 3984-3989.

44. Pearce, R.; Marchessault, R. H. *Polymer* **1994,** 35, 3990-3997.

45. Abe, H.; Doi, Y. *Macromolecules* **1994,** 27, 50-54.

46. Wallen, L. L.; Rohwedder, W. K. *Environ. Sci. Technol.* **1974,** 8, 576-579.

47. Findlay, R. H.; White, D. C. *Appl. Environ. Microbiol.* **1983,** 45, 71-78.

48. Capon, R. J.; Dunlop, R. W.; Ghisalberti, E. L.; Jefferies, P. R. *Phyto-chemistry* **1983,** 22, 1181-1184.

49. Odham, G.; Tunlid, A.; Westerdahl, G.; Marden, P. *Appl. Environ. Microbiol.* **1986,** 52, 905-910.

50. Holmes, P. A. *Phys. Technol.* **1985,** 16, 32-36.

51. Byrom, D. *Trends Biotechnol.* **1987,** 5, 246-250.

52. Doi, Y.; Kunioka, M.; Nakamura, Y.; Soga, K. *Macromolecules* **1987,** 20, 2988-2991.

53. Bloembergen, S.; Holden, D. A.; Hamer, G. K.; Bluhm, T. L.; Marchessault, R. H. *Macromolecules* **1986,** 19, 2865-2871.

54. Owen, A. J. *J. Colloid Polym. Sci.* **1985,** 263, 799.

55. Doi, Y.; Tamaki, A.; Kunioka, M.; Soga, K. *Appl. Microbio.. Biotechnol.* **1988,** 28, 330-334.

56. Doi, Y.; Tamaki, A.; Kunioka, M.; Soga, K. *J. Chem. Soc., Chem. Commun.* **1987**, 1635-1636.

57. Doi, Y.; Kunioka, M.; Nakamura, Y.; Soga, K. *Macromolecules* **1988,** 21, 2722-2727.

58. Kunioka, M.; Nakamura, Y.; Doi, Y. *Polymer Commun.* **1988,** 29, 174-176.

59. Kunioka, M.; Kawaguchi, Y.; Doi, Y. *Appl. Environ. Microbiol.* **1989,** 30, 569-573.

60. Doi, Y.; Segawa, A.; Kunioka, M. *Polymer Commun.* **1989,** 30, 169-171.

61. Kunioka, M.; Tamaki, A.; Doi, Y. *Macromolecules* **1989,** 22, 694-697.

62. Scandola, M.; Ceccorulli, G.; Doi, Y. *Int. J. Biol. Macromol.* **1990,** 12, 112-117.

63. Saito, Y.; Doi, Y. *Int. J. Biol. Macromol.* **1994,** 16, 99-104.

64. Saito, Y.; Nakamura, S.; Hiramitsu, M.; Doi, Y. *Polymer Int.* **1996,** 39, 169-174.

65. Doi, Y.; Segawa, A.; Kunioka, M. *Int. J. Biol. Macromol.* **1990,** 12, 106-111.

66. Shimamura, E.; Scandola, M.; Doi, Y. *Macromolecules* **1994,** 27, 4429-4435.

67. Doi, Y.; Kitamura, S.; Abe, H. *Macromolecules* **1995,** 28, 4822-4828.

68. Abe, H.; Doi, Y. *Int. J. Biol. Macromol.* **1994,** 16, 115-119.

69. Kemnitzer, J. E.; McCarthy, S. P.; Gross, R. A. *Macromolecules* **1993,** 26, 1221-1229.

70. Hori, Y.; Hagiwara, T. *Int. J. Biol. Macromol.* **1999,** 25, 237-245.

71. Kemnitzer, J. E.; McCarthy, S. P.; Gross, R. A. *Macromolecules* **1993,** 26, 6143-6150.

72. Amgoune, A.; Thomas, C. M.; Ilinca, S.; Roisnel, T.; Carpentier, J. *Angew Chem. Int. Ed.* **2006,** 45, 2782-2784.

73. Tanahashi, N.; Doi, Y. *Macromolecules* **1991,** 24, 5732-5733.

74. Rieth, L. R.; Moore, D. R.; Lobkovsky, E. B.; Coates, G. W. *J. Am. Chem. Soc.* **2002,** 124, 15239-15248.

75. Wu, B.; Lenz, R. W. *Macromolecules* **1998,** 31, 3473-3477.

76. Jaimes, C.; Collet, A.; Giani-Beaune, O.; Schue, F.; Amass, W.; Amass, A. *Polym. Int.* **1998,** 45, 5-13.

77. Wei, Z.; Liu, L.; Qi, M. *Eur. Polym. J.* **2007,** 43, 1210-1218.

78. Benvenuti, M.; Lenz, R. W. *J. Poly. Sci.: Poly. Chem.* **1991,** 29, 793-805.

79. Schue, F.; Jaimes, C.; Schue, R. D.; Giani-Beaune, O.; Amass, W.; Amass, A. *Polym. Int.* **2000,** 49, 965-974.

80. Arcana, M.; Giani-Beaune, O.; Schue, R.; Schue, F.; Amass, W.; Amass, A. *Polym. Int.* **2002,** 51, 859-866.

81. Wei, Z.; Liu, L.; Qi, M. *Reactive & Functional Polymers* **2006,** 66, 1411-1419.

82. Hirt, T. D.; Neuenschwander, P.; Suter, U. W. *Macromol. Chem. Phys.* **1996,** 197, 1609-1614.

83. Hirt, T. D.; Neuenschwander, P.; Suter, U. W. *Macromol. Chem. Phys.* **1996,** 197, 4253-4268.

84. Andrade, A. P.; Neuenschwander, P.; Hany, R.; Egli, T.; Witholt, B.; Li, Z. *Macromolecules* **2002,** 35, 4946-4950.

85. Andrade, A. P.; Witholt, B.; Chang, D.; Li, Z. *Macromolecules* **2003,** 36, 9830-9835.

86. Zhao, Q.; Cheng, G. *J. Mater. Sci.* **2004,** 39, 3829-3831.

87. Li, X.; Loh, X. J.; Wang, K.; He, C.; Li, J. *Biomacromolecules* **2005,** 6, 2740-2747.

88. Li, J.; Li, X.; Ni, X.; Leong, K. W. *Macromolecules* **2003,** 36, 2661-2667.

89. Liu, K. L.; Goh, S. H.; Li, J. *Polymer* **2008,** 49, 732-741.

90. Abe, H.; Doi, Y.; Kumagai, Y. *Macromolecules* **1994,** 27, 6012-6017.

91. Smith, A. L., *Oxford dictionary of biochemistry and molecular biology* Oxford University Press: Oxford, 1997.

92. Garrett, R. H.; Grisham, C. M., *Biochemistry*. Saunders College Pub: Philadelphia, 1999.

93. Jones, J. B. *Tetrahedron* **1986,** 42, 3351-3403.

94. Klibanov, A. M. *Acc. Chem. Res.* **1990,** 23, 114-120.

95. Santaniello, E.; Ferraboschi, P.; Grisenti, P.; Manzocchi, A. *Chem. Rev.* **1992,** 92, 1071-1140.

96. Wong, C. H.; Whitesides, G. M., *Enzymes in Synthetic Organic Chemistry*. Pergamon: Oxford, 1994.

97. Seoane, G. *Curr. Org. Chem.* **2000,** 4, 283-304.

98. Klibanov, A. M. *Trends Biotechnol.* **1997,** 15, 97-101.

99. Kobayashi, S.; Shoda, S.; Uyama, H. *Adv. Polym. Sci.* **1995,** 121, 1-30.

100. Kobayashi, S.; Shoda, S.; Uyama, H., *Precision Polymerization*. John Wiley & Sons: Chichester, 1997.

101. Gross, R. A.; Kaplan, D. L.; Swift, G., *ACS Symposium Series*. ACS: Washington, D.C., 1998; Vol. 684.

102. Kobayashi, S.; Uyama, H., *Materials Science and Technology-Synthesis of Polyemrs*. Wiley-VCH: Weinheim, 1998.

103. Kobayashi, S.; Uyama, H., *Encyclopedia of Polymer Science and Technology*. 3rd. ed.; Wiley: New York, 2001.

104. Kobayashi, S.; Uyama, H.; Kimura, S. *Chem. Rev.* **2001,** 101, 3793-3818.

105. Xie, Z. F. *Tetrahedron Asymmetry* **1991,** 2, 733-750.

106. Svendsen, A. *Biochim Biophys Acta* **2000,** 1543, 223-228.

107. Jaeger, K. E.; Ransac, S.; Dijkstra, B. W.; Colson, C.; van Heuvel, M.; Misset, O. *FEMS Microbial. Rev.* **1994**, 29-63.

108. Theil, F. *Chem. Rev.* **1995,** 95, 2203-2227.

109. Schmid, R. D.; Verger, R. *Angew Chem. Int. Ed.* **1998,** 37, 1608-1633.

110. Gotor, V. *Bioorg. Med. Chem.* **1999,** 7, 2189-2194.

111. Kobayashi, S.; Uyama, H., *Biopolyesters*. Springer-Verlag: Heidelberg, 2001; p 241-262.

112. Knani, D.; Gutman, A. L.; Kohn, D. H. *J. Poly. Sci.: Poly. Chem.* **1993,** 31, 1221-1232.

113. Uyama, H.; Kobayashi, S. *Chem. Lett.* **1993,** 22, 1149-1150.

114. Matsumura, S.; Beppu, H.; Tsukada, K.; Toshima, K. *Biotechnol. Lett.* **1996,** 18, 1041-1046.

115. Namekawa, S.; Uyama, H.; Kobayashi, S. *Polym. J.* **1996,** 28, 730-731.

116. Nobes, G. A. R.; Kazlauska, R. J.; Marchessault, R. H. *Macromolecules* **1996,** 29, 4829-4833.

117. Suzuki, Y.; Taguchi, S.; Hisano, T.; Toshima, K.; Matsumura, S.; Doi, Y. *Biomacromolecules* **2003,** 4, 537-543.

118. Matsumura, S.; Suzuki, Y.; Tsukada, K.; Toshima, K.; Doi, Y.; Kasuya, K. *Macromolecules* **1998,** 31, 6444-6449.

119. Dong, H.; Wang, H.; Cao, S.; Shen, J. *Biotechnol. Lett.* **1998,** 20, 905-908.

120. Kobayashi, S.; Takeya, K.; Suda, S.; Uyama, H. *Macromol. Chem. Phys.* **1998,** 199, 1729-1736.

121. MacDonald, R. T.; Pulapura, S. K.; Svirkin, Y. Y.; Gross, R. A.; Kaplan, D. L. *Macromolecules* **1995,** 28, 73-78.

122. Uyama, H.; Suda, S.; Kikuchi, H.; Kobayashi, S. *Chem. Lett.* **1997,** 26, 1109-1110.

123. Cordova, A.; Iversen, T.; Hult, K.; Martinelle, M. *Polymer* **1998,** 39, 6519-6524.

124. Srivastava, R. K.; Albertsson, A. C. *Macromolecules* **2006,** 39, 46-54.

125. Mei, Y.; Kumar, A.; Gross, R. A. *Macromolecules* **2003,** 36, 5530-5536.

126. Panova, A. A.; Kaplan, D. L. *Biotechnol. Bioeng.* **2003,** 84, 103-113.

127. Kobayashi, S.; Uyama, H.; Namekawa, S.; Hayakawa, H. *Macromolecules* **1998,** 31, 5655-5659.

128. Runge, M.; O'Hagan, D.; Haufe, G. *J. Poly. Sci.: Poly. Chem.* **2000,** 38, 2004.

129. Uyama, H.; Takeya, K.; Kobayashi, S. *Bull. Chem. Soc. Jpn.* **1995,** 68, 56-61.

130. Uyama, H.; Takeya, K.; Hoshi, N.; Kobayashi, S. *Macromolecules* **1995,** 28, 7046-7050.

131. Uyama, H.; Kikuchi, H.; Takeya, K.; Kobayashi, S. *Acta Polym.* **1996,** 47, 357-360.

132. Bisht, K. S.; Henderson, L. A.; Gross, R. A.; Kaplan, D. L.; Swift, G. *Macromolecules* **1997,** 30, 2705-2711.

133. Kumar, A.; Kalra, B.; Dekhterman, A.; Gross, R. A. *Macromolecules* **2000,** 33, 6303-6309.

134. Namekawa, S.; Uyama, H.; Kobayashi, S. *Proc. Japan Acad. Ser. B* **1998,** 74.

135. Kobayashi, S.; Uyama, H.; Namekawa, S. *Polym. Degrad. Stab.* **1998,** 59, 195-201.

136. Peeters, J. W.; van Leeuwen, O.; Palmans, A. R. A.; Meijer, E. W. *Macromolecules* **2005,** 38, 5587-5592.

137. Svirkin, Y. Y.; Xu, J.; Gross, R. A.; Kaplan, D. L.; Swift, G. *Macromolecules* **1996,** 29, 4591-4597.

138. Nishida, H.; Yamashita, M.; Nagashima, M.; Endo, T.; Tokiwa, Y. *J. Poly. Sci.: Poly. Chem.* **2000,** 38, 1560-1567.

139. MacDonald, R. T.; Pulapura, S. K.; Svirkin, Y. Y.; Gross, R. A. *Macromolecules* **1995,** 28, 73-78.

140. Kumar, A.; Gross, R. A. *Biomacromolecules* **2000,** 1, 133-138.

141. Dong, H.; Cao, S.; Li, Z.; Han, S.; You, D.; Shen, J. *J. Poly. Sci.: Poly. Chem.* **1999,** 37, 1265-1275.

142. Zacchigna, M.; Luca, G. D.; Lassiani, L.; Varnavas, A.; Boccu, E. *IL Farmaco.* **1998,** 53, 758-763.

143. Gross, R. A.; Kumar, A.; Kalra, B. *Chem. Rev.* **2001,** 101, 2097-2124.

144. Lanee, C.; Boeran, S.; Vos, K.; Veeger, C. *Biothechnol. Bioeng.* **1987,** 30, 81-87.

145. Gutman, A. L.; Zuobi, K.; Bravdo, T. *J. Org. Chem.* **1990,** 55, 3546-3552.

146. Mei, Y.; Kumar, A.; Gross, R. A. *Macromolecules* **2002,** 35, 5444-5448.

147. Matsumuto, M.; Odachi, D.; Kondo, K. *Biochem. Eng. J.* **1999,** 4, 73-76.

148. Cordova, A.; Iversen, T.; Hult, K. *Polymer* **1999,** 40, 6709-6721.

149. Srivastava, R. K.; Albertsson, A. C. *Macromolecules* **2006,** 39, 46-54.

150. Hedfors, C.; Ostmark, E.; Malmstrom, E.; Hult, K.; Martinelle, M. *Macromolecules* **2005,** 38, 647-649.

151. Henderson, L. A.; Svirkin, Y. Y.; Gross, R. A. *Macromolecules* **1996,** 29, 7759-7766.

152. Kawaguchi, T.; Nakano, M.; Juni, K. T.; Inoue, S.; Yoshida, Y. *Chem. Pharm. Bull.* **1983,** 31, 4157-4160.

153. Zhu, K. J.; Hendren, R. W.; Jensen, K.; Pitt, C. G. *Macromolecules* **1991,** 24, 1736-1740.

154. Kojimai, M.; Nakano, M.; Juni, K. T.; Inoue, S.; Yoshida, Y. *Chem. Pharm. Bull.* **1984,** 32, 2795-2802.

155. Matsumura, S.; Tsukada, K.; Toshima, K. *Macromolecules* **1997,** 30, 3122-3124.

156. Kobayashi, S.; Kikuchi, H.; Uyama, H. *Macromol. Rapid Commun.* **1997,** 18, 575-579.

157. Bisht, K. S.; Svirkin, Y. Y.; Henderson, L. A.; Gross, R. A. *Macromolecules* **1997,** 30, 7735-7742.

158. Bisht, K. S.; Deng, F.; Gross, R. A.; Kaplan, D. L.; Swift, G. *J. Am. Chem. Soc.* **1998,** 120, 1363-1367.

159. Deng, F.; Gross, R. A. *Int. J. Biol. Macromol.* **1999,** 25, 153-159.

160. Kricheldorf, H. R.; Stricker, A. *Macromol. Chem. Phys.* **1999,** 200, 1726-1733.

161. Kumar, A.; Garg, K.; Gross, R. A. *Macromolecules* **2001,** 34, 3527-3533.

162. Focarete, M. L.; Gazzano, M.; Scandola, M.; Kumar, A.; Gross, R. A. *Macromolecules* **2002,** 35, 8066-8071.

163. Al-Azemi, T. F.; Harmon, J. P.; Bisht, K. S. *Biomacromolecules* **2000,** 1, 493-500.

164. Al-Azemi, T. F.; Bisht, K. S. *J. Poly. Sci.: Poly. Chem.* **2002,** 40, 1267-1274.

165. Okumura, S.; Iwai, M.; Tsujisaka, Y. *J. Jpn. Oil Chem. Soc.* **1983,** 32, 271.

166. Gatfield, I. L. *Ann. N.Y. Acad. Sci.* **1984,** 434, 569-572.

167. Makita, A.; Nihira, T.; Yamada, Y. *Tetrahedron Lett.* **1987,** 28, 805-808.

168. Gutman, A. L.; Zuobi, K.; Boltansky, A. *Tetrahedron Lett.* **1987,** 28, 3861-3864.

169. Margolin, A. L.; Crenne, J. Y.; Klibanov, A. M. *Tetrahedron Lett.* **1987,** 28, 1607-1609.

170. Guo, Z.; Sih, C. J. *J. Am. Chem. Soc.* **1988,** 110, 1999-2001.

171. Okumura, S.; Iwai, M.; Tominaga, K. *Agric. Biol. Chem.* **1984,** 48, 2805-2808.

172. Linko, Y. Y.; Wang, Z.; Seppala, J. *J. Biotechnol.* **1995,** 40, 133-138.

173. Wang, Z.; Hiltunen, K.; Orava, P.; Seppala, J.; Linko, Y. Y. *J. Macromol. Sci.: Pure Appl. Chem.* **1996,** 33, 599-612.

174. Wu, X.; Linko, Y. Y.; Seppala, J. *Ann. N.Y. Acad. Sci.* **1998,** 864, 399-404.

175. Uyama, H.; Inada, K.; Kobayashi, S. *Chem. Lett.* **1998,** 27, 1285-1286.

176. Mahapatro, A.; Kalra, B.; Kumar, A.; Gross, R. A. *Biomacromolecules* **2003,** 4, 544-551.

177. Sahoo, B.; Bhattacharya, A.; Fu, H.; Gao, W.; Gross, R. A. *Biomacromolecules* **2006,** 7, 1042-1048.

178. Kobayashi, S.; Uyama, H.; Suda, S.; Namekawa, S. *Chem. Lett.* **1997,** 26, 105-105.

179. Uyama, H.; Takamoto, T.; Kobayashi, S. *Polym. J.* **2002,** 34, 94-96.

180. Nara, S. J.; Harjani, J. R.; Salunkhe, M. M.; Mane, A. T.; Wadgaonkar, P. P. *Tetrahedron Lett.* **2003,** 44, 1371-1373.

181. Nakaoki, T.; Danno, M.; Kurokawa, K. *Polym. J.* **2003,** 35, 791-797.

182. Geresh, S.; Gilboa, Y. *Biotech. Bioeng.* **1990,** 36, 270-274.

183. Wallace, J. S.; Morrow, C. J. *J. Poly. Sci.: Poly. Chem.* **1989,** 27, 3271-3284.

184. Mezoul, G.; Lalot, T.; Brigodiot, M.; Narecgakm, E. *J. Poly. Sci.: Poly. Chem.* **1995,** 33, 2691-2698.

185. Gryglewicz, S. *J. Mol. Catal. B Enz.* **2001,** 15, 9-13.

186. Lavalette, A.; Lalot, T.; Brigodiot, M.; Marechal, E. *Biomacromolecules* **2002,** 3, 225-228.

187. Mezoul, G.; Lalot, T.; Brigodiot, M.; Marechal, E. *Polym. Bull.* **1996,** 36, 541-548.

188. Linko, Y. Y.; Seppala, J. *CHEMTECH* **1996,** 26, 25-31.

189. Wallace, J. S.; Morrow, C. J. *J. Poly. Sci.: Poly. Chem.* **1989,** 27, 2553-2567.

190. Morrow, C. J. In *Biocatalytic Synthesis of Polyesters Using Enzymes*, MRS Bull. 1992 Nov., pp 43-47.

191. Chaudhary, A. K.; Beckman, E. J.; Russell, A. J. *J. Am. Chem. Soc.* **1995,** 117, 3728-3733.

192. Linko, Y. Y.; Wang, Z.; Seppala, J. *Enzyme Microb. Technol.* **1995,** 17, 506-511.

193. Wang, Y. F.; Lalonde, J. J.; Momongan, M.; Bergbreiter, D. E.; Wong, C. H. *J. Am. Chem. Soc.* **1988,** 110, 7200-7205.

194. Chaudhary, A. K.; Lopez, J.; Beckman, E. J.; Russell, A. J. *Biotech. Prog.* **1997,** 13, 318-325.

195. Pavel, K.; Ritter, H., *Chemoenzymatic Synthesis and Modification of Monomers and Polymers*. ACS: Washington DC, 1996; p 200.

196. Chaudhary, A. K.; Beckman, E. J.; Russell, A. J. *Biotechnol. Bioeng.* **1998,** 59, 428-437.

197. Kobayashi, S. *J. Poly. Sci.: Poly. Chem.* **1999,** 37, 3041-3056.

198. Uyama, H.; Kobayashi, S. *J. Mol. Catal. B Enz.* **2002,** 19-20, 117-127.

199. Albertsson, A. C.; Varma, I. K. *Biomacromolecules* **2003,** 4, 1466-1486.

200. Namekawa, S.; Uyama, H.; Kobayashi, S. *Biomacromolecules* **2000,** 1, 335-338.

201. Jedlinski, Z.; CKowalczuk, M.; Adamus, G.; Sikorska, W.; Rydz, J. *Int. J. Biol. Macromol.* **1999,** 25, 247-253.

202. Kikuchi, H.; Uyama, H.; Kobayashi, S. *Macromolecules* **2000,** 33, 8971-8975.

203. Griffith, L. G. *Acta Mater.* **2000,** 48, 263-277.

204. Engelberg, I.; Kohn, J. *Biomaterials* **1991,** 12, 292-304.

205. Vert, M.; Mauduit, J.; Li, S. *Biomaterials* **1994,** 15, 1209-1213.

206. Schmitt, E. A.; Flanagan, D. R.; Linhardt, R. J. *Macromolecules* **1994,** 27, 743-748.

207. Pitt, C. G., *Biodegradable Polymers as Drug Delivery System*. Marcel Dekker: New York, 1990; p 71-120.

208. Gopferich, A. *Biomaterials* **1996,** 17, 103-114.

209. Gomes, M. E.; Reis, R. L. *Int. Mater. Rev.* **2004,** 49, 261-273.

210. Pachence, J. M.; Kohn, J., *Principles of tissue engineering*. Academic Press: New York, 1997.

211. Zhang, R.; Ma, P. X. *J. Biomed. Mater. Rev.* **1999,** 44, 446-455.

212. Domb, A. J.; Kost, J.; Wiseman, D. M., *Handbook of Biodegradable Polymers*. Harwood: Amsterdam, 1997.

213. Kohn, J.; Langer, R., *Biomaterials Science*. Academic Press: New York, 1996.

214. Middleton, J. C.; Tipton, A. J. *Biomaterials* **2000,** 21, 2335-2346.

215. Ratner, B. D.; Hoffman, A. S.; Schoen, F. J.; Lemons, J. E., *Biomaterials Science: An Introduction to Materials in Medicine.* 2nd. ed.; Elsevier: San Diego, 2004.

216. Pego, A. P.; Grijpma, D. W.; Feijen, J. *Polymer* **2003,** 44, 6495-6504.

217. Pego, A. P.; Poot, A. A.; Girjpma, D. W.; Feijen, J. *J. Mater. Sci.: Mater. Med.* **2003,** 14, 767-771.

218. Zhang, Z.; Grijpma, D. W.; Feijen, J. *Macromol. Chem. Phys.* **2004,** 205, 867-875.

219. Sudesh, K.; Doi, Y., *Handbook of Biodegradable Polymers*. Rapra: Shawbury, 2005; p 219.

220. Impallomeni, G.; Giuffrida, M.; Barbuzzi, T.; Musumarra, G.; Ballistreri, A. *Biomacromolecules* **2002,** 3, 835-840.

221. Kumar, A.; Gross, R. A. *J. Am. Chem. Soc.* **2000,** 122, 11767-11770.

222. Chen, C.; Fei, B.; Peng, S. *J. Poly. Sci.: Poly. Phys.* **2002,** 40, 1893-1903.

223. Kwwaguchi, T.; Nakano, M.; Juni, K. T.; Inoue, S.; Yoshida, Y. *Chem. Pharm. Bull.* **1983,** 31, 4157-4160.

224. Schindler, A.; Jeffcoat, R.; Kimmel, G. L.; Pitt, C. G.; Wall, M. E.; Zweidinger, R. A., *Contemporary Topics in Polymer Sciences*. New York, 1997.

225. Zhang, Z.; Girjpma, D. W.; Feijen, J. *J. Control. Release* **2006,** 112, 57-63.

226. Darensbourg, D. J.; Ganguly, P.; Billodeaux, D. *Macromolecules* **2005,** 38, 5406-5410.

227. Darensbourg, D. J.; Choi, W.; Ganguly, P.; Richers, C. P. *Macromolecules* **2006,** 39, 4374-4379.

228. Pospiech, D.; Komber, H.; Jehnichen, D.; Haussler, L.; Eckstein, K.; Scheibner, H.; Janke, A.; Kricheldorf, H. R.; Petermann, O. *Biomacromolecules* **2005,** 6, 439-446.

229. Varma, I. K.; Albertsson, A. C.; Rajkhowa, R.; Srivastava, R. K. *Prog. Polym. Sci.* **2005,** 30, 949-981.

230. Feng, J.; He, F.; Zhuo, R. *Macromolecules* **2002,** 35, 7175-7177.

231. Bat, E.; Plantinga, J. A.; Harmsen, M. C.; van Luyn, M. J. A.; Zhang, Z.; Grijpma, D. W.; Feijen, J. *Biomacromolecules* **2008,** 9, 3208-3215.

232. Sudesh, K.; Abe, H.; Doi, Y. *Prog. Polym. Sci.* **2000,** 25, 1503-1555.

233. Zinn, M.; Witholt, B.; Egli, T. *Adv. Drug. Deliv. Rev.* **2001,** 53, 5-21.

234. Valappil, S. P.; Misra, S. K.; Boccaccini, A. R.; Roy, I. *Expt. Rev. Med. Dev.* **2006,** 3, 853-868.

235. Li, X.; Zhang, Y.; Chen, G. *Biomaterials* **2008,** 29, 3720-3728.

236. Dai, S.; Li, Z. *Biomacromolecules* **2008,** 9, 1883-1893.

237. Uyama, H.; Kobayashi, S. *Adv. Polym. Sci.* **2006,** 194, 133-158.

238. Marechal, E. *Curr. Org. Chem.* **2002,** 6, 177-208.

239. Uyama, H.; Kobayashi, S. *Chem. Lett.* **1994,** 23, 1687-1690.

240. Uyama, H.; Yaguchi, S.; Kobayashi, S. *J. Poly. Sci.: Poly. Chem.* **1999,** 37, 2737-2745.

241. Linko, Y. Y.; Lamsa, M.; Wu, X.; Uosukanum, E.; Seppala, J.; Linko, P. *J. Biotechnol.* **1998,** 66, 41-50.

242. Matsumura, S. *Macromol. Biosci.* **2002,** 2, 105-126.

243. Uyama, H.; Inada, K.; Kobayashi, S. *Polym. J.* **2000,** 32, 440-443.

244. Bordes, P.; Pollet, E.; Averous, L. *Prog. Polym. Sci.* **2009,** 34, 125-155.

245. Reeve, M. S.; McCarthy, S. P.; Gross, R. A. *Macromolecules* **1993,** 26, 888-894.

246. Chen, G. Q. *Chem. Soc. Rev.*, **2009**, *38*, 2434.

247. Hocking, P. J.; Marchessault, R. H. In *Chemistry and Technology of Biodegradable Polymers*; Griffin, G. J. L., Ed.; Blackie: Glasgow, 1994; Chapter 4, p 48.

248. Holmes, P. A. *Phys. Technol.* **1985**, *16*, 32.

249. Doi, Y. *Macromol. Symp.* **1995**, *98*, 585.

250. Sudesh, K.; Abe, H.; Doi, Y. *Prog. Polym. Sci*, **2000**, *25*, 1503.

251. Zinn, M.; Witholt, B.; Egli, T. *Adv. Drug Deliv. Rev.*, **2001**, *53*, 5.

252. Sudesh, K.; Doi, Y. *Handbook of Biodegradable Polymers*, Shawbury: Rapra, 2005.

252. Valappil, S. P.; Misra, S. K.; Boccaccini, A. R.; Roy, I. *Expt. Rev. of Med. Dev.*, **2006**, *3*, 853.

254. Li, X.; Zhang, Y.; Chen, G. *Biomaterials*, **2008**, *29*, 3720.

255. Moore, T.; Adhikari, R.; Gunatillake, P. *Biomaterials* **2005**, *26*, 3771.

256. Reeve, M. S.; McCarthy, S. P.; Gross, R. A. *Macromolecues* **1993**, 2*6*, 888.

257. Marchessault, R. H.; Monasterios, C. J.; Morin, F. G.; Sundararajan, P. R. *Int. J. Biol. Macromol.* **1990**, *12*, 158.

258. Witholt, B.; Kessler, B. *Curr. Opin. Biotechnol.* **1999**, *10*, 279.

259. Preusting, H.; Nijenhuis, A.; Witholt, B. *Macromolecules*, **1990**, *23*, 4220.

260. Gross, R. A.; DeMello, C.; Lenz, R. W.; Brandl, H.; Fuller, R. C. *Macromolecules* **1989**, *22*, 1106.

261. Kato, M.; Fukui, T.; Doi, Y. *Bull. Chem. Soc. Jpn.* **1996**, *69*, 515.

262. Puskas, J. E.; Chen, Y. *Biomacromolecues* **2004**, *5*, 1141.

263. Schindler, A.; Jeffcoat, R.; Kimmel, G. L.; Pitt, C. G.; Wall, M. E.; Zweidinger, R. A. *Contemporary Topics in Polymer Sciences*. New York: Plenum, 1997.

264. Zhang, Z.; Girjpma, D. W.; Feijen, J. *J. Control. Release*, **2006**, *112*, 57.

265. Kricheldorf, H. R.; Stricker, A. *Macromol. Chem. Phys.*, **1999**, *200*, 1726.

266. Pospiech, D.; Komber, H.; Jehnichen, D.; Haussler, L.; Eckstein, K.; Scheibner, H.; Janke, A.; Kricheldorf, H. R.; Petermann, O. *Biomacromolecues*, **2005**, *6*, 439.

267. Varma, I. K.; Albertsson, A.-C.; Rajkhowa, R.; Srivastava, R. K. *Prog. Polym. Sci.*, **2005**, *30*, 949.

268. Andronova, N.; Albertsson, A.-C. *Biomacromolecules* **2006**, *7*, 1489-1495.

269. Jiang, Z.; Azim, H.; Gross, R. A. *Biomacromolecules* **2007**, *8*, 2262-2269.

270. Srivastava, R. K.; Albertsson, A.-C. *Macromolecules* **2007**, *40*, 4464-4469.

271. Hakkarainen, M.; Adamus, G.; Hoeglund, A.; Kowalczuk, M.; Albertsson, A.-C. *Macromolecules* **2008**, *41*, 3547-3554.

271. Hans, M.; Gasteier, P.; Keul, H.; Moeller, M. *Macromolecules* **2006**, *39*, 3184-3193.

272. Andrade, A. P.; Witholt, B.; Hany, R.; Egli, T.; Li, Z. *Macromolecules* **2002**, *35*, 684-689.

List of Publications

Publications:

1. **S. Dai** and Z. Li, Enzymatic preparation of novel thermoplastic *di*-block copolyesters containing poly[(R)-3-hydroxybutyrate] and poly(*epsilon*-caprolactone) blocks *via* ring-opening polymerization. *Biomacromolecules,* 2008, 9(7) 1883-1893.

2. L. Xue, **S. Dai** and Z. Li, Synthesis and characterization of three-arm poly(*epsilon*-caprolactone)-based poly(ester-urethanes) with shape-memory effect at body temperature. *Macromolecules*, 2009, 42(4) 964-972.

3. **S. Dai,** L. Xue and Z. Li, Enzyme-Catalyzed Polycondensation of Polyester Macrodiols with Divinyl Adipate: A Green Method for the Preparation of Thermalplastic Block Copolyesters, *Biomacromolecules,* 2010, 10, 3176-3181.

4. **S. Dai,** L. Xue and Z. Li, Enzymatic Ring-Opening Polymerization of Trimethylene Carbonate With Polyester Macro-diols: Preparation of *di*- or *tri*-Blcok Hydroxylated Poly(ester-carbonate)s. (submitted to *Macromolecules*)

Presentations:

1. **S. Dai,** and Z. Li, Preparation of Novel Thermoplastic di-Block Copolyester Containing Poly[(R)-3-hydroxybutyrate] and Poly(-Caprolactone) Blocks *via* Enzymatic Ring-opening Polymerization. *Poster Presentation*, 8th. International Symposium on Biocatalysis and Biotransformations, Oviedo, Spain, Jul. 8-13, 2007.

2. **S. Dai,** L. Xue and Z. Li, Highly Selective Enzymatic Ring-Opening Polymerization: Syntheses and Characterizations of Thermoplastic Di-Block Co-Polyesters Containing Poly[(R)-3-Hydroxybutyrate] and Poly(å-Caprolactone) Blocks. *Oral Presentation*, AIChE Annual Meeting, Philadelphia, USA, Nov. 16-20, 2008.

3. **S. Dai,** L. Xue and Z. Li, Enzymatic Preparation and Characterization of di-Block Copolyester-carbonates Consisting of Poly[(R)-3-hydroxybutyrate] and Poly(trimethylene carbonate) Blocks *via* Ring-opening Polymerization. *Oral Presentation*, ACS 238th. National Meeting, Washington, D.C., USA, Aug. 16-20, 2009.